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(54) Title: A SOYBEAN PEROXIDASE GENE FAMILY AND AN ASSAY FOR DETECTING SOYBEAN PEROXIDASE ACTIVITY

(57) Abstract

Four cDNA sequences representing a soybean peroxidase gene family are provided. An enzyme-capture assay for the nondestructive, sensitive and reliable quantitation of peroxidase activity is also provided. Cultivars having a high-peroxidase level can be efficiently selected, providing a large, renewable source of peroxidase for use in industry and in diagnostic chemistries.

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A SOYBEAN PEROXIDASE GENE FAMILY AND AN ASSAY FOR DETECTING SOYBEAN PEROXIDASE ACTIVITY

Background of the Invention

The present invention relates to the DNA sequences of the soybean peroxidase, and to the enzymatic assay of peroxidase activity. The invention further relates to medical and environmental diagnostics employing soybean peroxidase monoclonal antibody in place of horseradish peroxidase polyclonal antibodies which has been historically used.

Peroxidase is a class of proteins whose primary function is to oxidize a variety of hydrogen donors at the expense of peroxide or molecular oxygen. Areas where peroxidase could have an immediate use are: pulp and paper bleaching; on-site waste destruction; soil remediation; organic synthesis; and diagnostic chemistries.

At present, pulp and paper is bleached using chloride ions as a chemical agent. Soybean peroxidase has several advantages over chlorine bleach: lower cost; environmentally friendly; and hydroxyl ions produced by peroxidase have twice the oxidation power of chlorine ions.

In waste water and soil treatments, peroxidase has advantages since many organic compounds are toxic, inhibitory, or refractory to microbes, and certain organic compounds may result in the production of microbial products that produce toxic or offensive effluent.

The use of oxidation to achieve on-site destruction or detoxification of contaminated water and waste will increase in the future. If carried out to its ultimate

stage, oxidation can completely oxidize organic compounds to carbon dioxide, water and salts.

Peroxidase has several uses in organic synthesis. Using peroxidase, researchers synthesized conductive polyaniline that produced only water as a by-product. Peroxidase can also be used in the manufacturing of adhesive and antioxidant intermediates.

Enzymes are now widely used in medical and environmental diagnostics. Horseradish peroxidase has been one of the most satisfactory enzymes but is relatively expensive. It has now been found that soybean peroxidase can be readily harvested from soybean hulls at minimal expense and be substituted for horseradish peroxidase in these diagnostic chemistries.

Several diagnostic chemistries using the enzymatic activity of horseradish peroxidase and polyclonal antibodies have been described in the literature. Horseradish peroxidase has been used for diagnostic determinations of various analytes and has been used as a label in enzyme labeled antibodies used in the determination of immunologically reactive species (i.e., immunoassays). Such determinations can be carried out in solution or in dry analytical elements.

One type of useful assay utilizes enzymatic reactions wherein the analyte, upon contact with the appropriate reagents, reacts with oxygen in the presence of a suitable enzyme to produce hydrogen peroxide in proportion to the concentration of the analyte. A detectable product such as a visible or fluorescent dye is then produced by the reaction of hydrogen peroxide in proportion to the concentration of the analyte in the tested liquids. Peroxidase is generally used in such assays to catalyze the oxidation of the interactive composition by hydrogen peroxide. One example of such an assay is a glucose assay using glucose oxidase. Glucose is oxidized in the presence of oxygen by the enzyme, glucose oxidase, to produce glucolactone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide oxidizes a colorless dye such as tetramethylbenzidine to produce a colored product.

Another type of assay utilizes an immunologically reactive compound such as an antibody. These chemistries can be generally classified into two groups, namely, conjugate or enzyme labeled antibody procedures, and non-conjugate or unlabeled antibody procedures. In the conjugate procedures, the enzyme is covalently linked to the antibody and applied to a sample containing the immobilized antigen to be detected. Thereafter the enzyme substrate, e.g., hydrogen peroxide, and an oxidizable chromogen such as a leuco dye are applied. In the presence of the peroxidase, the peroxide reacts with the chromogen resulting in the production of color. The production of color indicates the presence and in some cases the amount of the antigen. In another method, a competing substance is used to dislodge an antibody enzyme conjugate from an immobilized substrate, leading to an absence of color.

In a method sometimes referred to as the sandwich assay or enzyme linked immunoadsorbent assay (ELISA), a first antibody is bound to a solid support surface and contacted with a fluid sample suspected to contain the antigen to be detected and an enzyme-antibody conjugate. The antigen complexes with the antibody and the conjugate bonds to the antigen. Subsequent introduction of the substrate and chromogen produces a visual indication of the presence of the antigen.

Procedures employing non-conjugated enzymes include the enzyme bridge method and the peroxidase-antiperoxidase method. These methods use an antiperoxidase antibody produced by injecting peroxidase into an animal such as a goat, rabbit or guinea pig. The method does not require chemical conjugation of the antibody to the enzyme but consists of binding the enzyme to the antigen through the antigen-antibody reaction of an immunoglobulin-enzyme bridge. In the enzyme bridge method a secondary antibody acts as an immunologic bridge between the primary antibody against the suspected antigen and the antiperoxidase antibody. The antiperoxidase antibody in turn binds the peroxidase which catalyzes the indicator reaction. In the peroxidase-antiperoxidase method, a complex of the peroxidase and

the antiperoxidase antibody is formed. This complex can then be used in the immunologic bridge method.

Though peroxidase genes from different biologic sources have been identified, including other plant peroxidase genes from horseradish, tomato, pea, arabidopsis, peanut and turnip, and bacterial lignin peroxidase gene, there have not been any reports regarding identification of peroxidase genes from soybean.

Soybean coats are abundant and inexpensive, making them an excellent source of peroxidase. Therefore, there is substantial interest in cloning soybean peroxidase genes which will open the possibility of characterization of the expression patterns of individual peroxidase isoforms during normal plant development and genetic and molecular manipulations for increased peroxidase activity.

Brief Description of the Drawings

Fig. 1 Average ELISA absorbance (405 nm) of purified peroxidase samples against 1:10 dilution of peroxidase monoclonal antibodies (MAB).

Fig. 2 Average Peroxidase Capture Assay (PCA absorbance (450 nm) of purified peroxidase samples against 1:5000 dilution of peroxidase MAB.

Fig. 3 Average guaiacol absorbance (470 nm) of purified peroxidase.

Fig. 4 Average PCA absorbance (450 nm) of peroxidase solutions of known activity against 1:5000 dilution of peroxidase MAB.

Fig. 5 Comparisons of nucleotide sequences of the coding regions of the *SEP*a1 and *SEP*a2 genes and the predicted amino acid sequences of *SEP*a1 *(p1)* and *SEP*a2 *(p2)*. Amino acid sequences are shown using the single-letter code. The complete coding and predicted amino acid sequences are given only for *SEP*a1 (first and third lines, respectively). To emphasize the similarity between the two genes and their products, only those nucleotides in the coding region of *SEP*a2 and the predicted amino acid that differ from the corresponding ones in *SEP*a1 and *p1* are shown. The dots indicate identity of nucleotides and amino acids. For

example, a dot under a nucleotide represents the presence of the same nucleotide that is directly above the dot. The signal peptide is shown in bold italics. The start of the mature proteins begins with the [QLXXXFY] motif at position 1. The cysteine residues in disulfide bridges are shaded. Conserved amino acid areas are outlines.

Fig. 6 Comparisons of the nucleotide sequences of the coding regions of the *SEP*b1 and *SEP*b2 genes and the predicted amino acid sequences of *SEP*b1 *(p3)* and *SEP*b2 *(p4)*. Amino acid sequences are shown using the single-letter code. The complete coding and predicted amino acid sequences are given only for *SEP*b1 (first and third lines, respectively). The dots indicate identity of nucleotides and amino acids. The asterisks indicate the gap of nucleotides and amino acids between *SEP*b1 and *SEP*b2, *p3* and *p3*, respectively. The cysteine residues are shaded and the conserved amino acid areas are outlines. For example, a dot under a nucleotide represents the presence of the same nucleotide that is directly above the dot. The signal peptide is shown in bold italics.

Fig. 7 Histogram of average SPCA absorbance of cultivars.

Fig. 8 Histogram of average absorbance of genotypes within an F_3 segregating population. Optical density values were 0.777 for Resnik and 0.502 for Winchester.

Summary of the Invention

The present invention relates to a method for quantifying plant peroxidase activity by using a monoclonal antibody against peroxidase.

The method of the present invention further allows a direct quantitative assay of peroxidase activity in biological materials and in solutions containing peroxidase.

Additionally, the method of the present invention can be used to identify differences in peroxidase activity between plant genotypes within a segregating population of genotypes, as in a plant breeding research field, grain elevator or

processing plant. Therefore, the method of the instant invention can be used to easily find and select for plants having improved levels of peroxidase activity. The invention is non-destructive to seed or plants. Cultivars selected using the method of the present invention increase the sensitivity of diagnostic applications and reduces the cost of enzyme purification.

The present invention further involves four DNA sequences representing a soybean peroxidase gene family. These DNA sequences of the present invention encode amino acids that show homology to other plant peroxidase conserved amino acid regions. Outside the conserved regions the sequences show a high degree of divergence from other plant peroxidases.

The amino acid sequences of the present invention further contain hydrophobic signal peptides at their N-termini and mature proteins can be secreted through all membranes.

The present invention further relates to using tetramethylbenzadine as a substrate, a simple linear model quantifies the relation between peroxidase activity and peroxidase quantity where the slope indicates the specific activity.

The method of the present invention further relates to a direct method without the secondary enzyme-linked antibody as used in reaction found in ELISA.

The invention also relates to a kit for measuring peroxidase activity outside the laboratory to determine the effect of environment and seed storage on peroxidase activity, and allows direct selection of high peroxidase genotypes in a plant breeding field, grain elevator and processing plant. The kit also allows quantitation and monitoring of peroxidase activity in processes using peroxidase or peroxidase solutions, such as pulp and paper bleaching, on-site waste destruction, soil remediation and organic synthesis.

The present invention also relates to an antiperoxidase antibody which does not inhibit peroxidase activity which can be used in the following: enzyme capture assay for activity quantification; ELISA for peroxidase concentration; soybean peroxidase capture assay (SPCA) kits for measuring activity outside the lab; ELISA kits for

measuring concentration outside the lab; peroxidase-antiperoxidase conjugates; immunohistochemical detection; immunoperoxidase microscopy and immunopurification of peroxidase.

The peroxidase-antiperoxidase conjugates of the present invention are useful in the following applications: non-radioactive nucleic acid labeling and detection; conjugating antibody complex in western blot; ELISA reactions; ELISA detection of DNA and RNA; and conjugate to polymerase chain reaction (PCR) products.

Detailed Description of the Invention

In order to provide an understanding of several of the terms used in the specification and claims, the following definitions are provided:

"Operably linked" - The term operably linked refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner, i.e., a promoter is operably linked to a coding sequence if the promoter affects its transcription or expression.

"Isolated", "substantially pure" and **"substantially homogeneous"** - These terms are used interchangeably to describe a protein or polypeptide which has been separated from components which accompany it in its natural state. A monomeric protein is substantially pure when at least about 60 to 75% of a sample exhibits a single polypeptide sequence. A substantially pure protein will typically comprise about 60 to 90% W/W of a protein sample, more usually about 95% w/w, and preferably will be over about 99% pure. Protein purity or homogeneity may be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein sample, followed by visualizing a single polypeptide band upon staining the gel. For certain purposes, higher resolution may be provided by using HPLC or other means well known in the art for purification utilized.

A MTS protein is substantially free of naturally associated components when it is separated from the native contaminants which accompany it in its natural state. Thus, a polypeptide which is chemically synthesized or synthesized in a cellular system different from the cell from which it naturally originates will be substantially

free from its naturally associated components. A protein may also be rendered substantially free of naturally associated components by isolation, using protein purification techniques well known in the art.

A polypeptide produced as an expression product of an isolated and manipulated genetic sequence is an "isolated polypeptide," as used herein, even if expressed in a homologous cell type. Synthetically made forms or molecules expressed by heterologous cells are inherently isolated molecules.

"Nondestructive" - The term nondestructive refers to the ability of quantitating peroxidase activity without killing the seed, plant or rendering peroxidase non-enzymatically active.

The present invention is directed to a method of quantitating peroxidase activity, a kit for quantitating peroxidase activity, immunological assays, and DNA sequences regulating and representing a soybean peroxidase gene family.

The method of this invention is adaptable to both solution and dry assays and describes the capture of peroxidase by an antibody from a solution. Antibodies are immobilized on a solid support and unbound matrix is blocked with unreactive proteins. Solutions containing peroxidase are incubated with the immobilized antibodies and then removed. Captured peroxidase is then assayed for activity with any substrate, with or without additives, previously used in horseradish peroxidase assays. This invention does not use a secondary enzyme-linked antibody like an ELISA assay.

The method of this invention can also be practiced with a dry analytical element. The kit may be composed of an absorbent carrier material, e.g. a thin sheet of a self-supporting absorbent or bibulous material, such as filter paper or strips, which contains an immobilized antibody. The element can be divided into multiple zones with different compositions of the antibody incorporated into individual zones of the carrier material. Such elements are known as test strips, diagnostic elements, dip sticks, diagnostic agents and the like.

The assay or test kit can be used to quantitate peroxidase activity in plant fluids from macerated tissue with or without exogenous liquid added. Such fluids include, but are not limited to, fluids from leaves, stems, roots, flowers, seeds, seed coats, embryos, hypocotyls, coleoptiles, seed pods and seed buds. It is also possible to assay fluids from a variety of plant species including, but not limited to, soybean, corn, wheat, sorghum and oats.

This invention allows for the selection of high peroxidase plant genotypes in the field of plant breeding. Since minimal amounts of tissue are needed, unlike other methods of assaying peroxidase activity, e.g. Gilliken and Graham, Plant Physiol. 96:214-220 (1991), this invention is non-destructive to the seed or resulting plant. This greatly accelerates the progress of plant breeding for high peroxidase levels. The non-destructive nature allows high peroxidase plant genotypes to be selected and advanced to the next generation. The non-destructive nature of the assay is unique. In addition to the non-destructive nature of the assay, another unique trait of the present invention is the quantitative nature of the assay. Being quantitative, the present invention allows for the ultimate discriminatory assay for the separation of high peroxidase genotypes. Previous assays are not able to separate high peroxidase genotypes, e.g. Buttery & Buzzell, Crop Science 8:722-725 (1968). The ranking of high peroxidase genotypes, based on activity, will allow for the most efficient selection for high peroxidase genotypes. This invention is unique in that it is the only method that is non-destructive to the seed or plant and also is quantitative.

The assay or kit can be used to monitor peroxidase activity in industrial processes and is an identity preserved system to deliver high peroxidase plant material to processors. In an identity preserved system, kits will be used to identify high peroxidase seeds or to monitor activity from the seed company, to the farmer's field, grain elevator, grain truck and finally to the processing facility. The kit also can be used to monitor peroxidase activity in stored peroxidase solutions. In industrial processes that use peroxidase, the kit can be used to monitor peroxidase activity.

The invention also can be used to determine antigens using an enzyme-antibody conjugate method. In this embodiment, the enzyme label can be any plant peroxidase that participates in the conversion of a chromogen or luminal to a detectable form.

Other uses of the present invention involve the modification of the peroxidase enzyme, the peroxidase gene or bacteria containing the enzyme. The entire gene with its 5'- and 3'- regulatory regions can be manipulated in a variety of ways to provide for expression and enzyme form.

In general, expression can be enhanced by including multiple copies of the peroxidase gene in a transformed bacterial or plant host, by using promoters that initiate transcription at increased levels, or by any known means of enhancing peptide expressions.

A recombinant gene can be constructed that takes advantage of regulatory regions from other genes and the coding region of the peroxidase genes. Alternatively, a recombinant gene can be constructed that takes advantage of the peroxidase regulatory regions and coding regions from other genes.

Examples

The following examples are provided to further illustrate the present invention and are not intended to limit the invention beyond the limitations set forth in the appended claims.

Example 1

Peroxidase Extraction and Monoclonal Antibody Production

Peroxidase was extracted from circular pieces of seed coat, roughly 3 mm in diameter. Samples from three seeds per replication were placed separately in micro centrifuge tubes containing 1 ml of water, incubated at room temperature for 2 hours and vortexed.

Purified seed coat peroxidase (>95% pure) and seed coat peroxidase solutions with various levels of known pupurogallin (PPU) activity were kindly provided by Enzymol International (Columbus, OH).

Seeds of high and low peroxidase cultivars were grown at the Purdue Agronomy Farm at West Lafayette, and a Resnik x Winchester cross was made during the summer of 1993. F_1 seeds were grown in Puerto Rico, F_2 seeds were grown in West Lafayette and F_3 individual seeds were tested for peroxidase activity.

BALB/c mice (*Mus musculus*) were subcutaneously injected with a total of 0.1 mg purified seed coat peroxidase (>95% pure) kindly provided by Mead Central Research (Chillicothe, OH). Fusions with myeloma parent P3/NS1/1-Ag4-1 (NS-1) were done with polyethylene glycol 4000. Hybridomas were selected on hypoxanthine (100 n*M*), aminopterin (0.4 n*M*), and thymidine (16 n*M*) media and clones were obtained using the limited dilution method. Raw ascites solution was collected and used in all procedures. Hybridomas were initially selected on their antibody's ability to bind peroxidase. Hybridomas were subsequently selected on their antibody's ability to bind peroxidase in such a way as to not affect enzymatic ability. We have selected a hybridoma that has been designated A4.

Example 2

Enzyme-linked Immunosorbent Assay (ELISA)

An indirect detection method using an alkaline phosphatase antimouse immunoglobulin and p-nitrophenyl phosphate as the chromogen was used to detect seed coat peroxidase. Raw ascites was diluted 1:10, 1:100, 1:1000, and 1:5000. Quantitation of three wells per replication was done at 405 nm after 45 minutes of development. ELISA detects protein or enzyme concentration but not enzyme activity, so ELISA is not suitable for plant breeding for higher peroxidase activity, or the detection or monitoring of peroxidase activity (Fig. 1)

Example 3

Peroxidase Capture Assay (PCA)

ELISA plate wells were coated with 100 μL of a 1:100, 1:1000, 1:5000, and 1:10,000 dilution of ascites fluid and incubated overnight at 4°C. After incubation, the ascites fluid was removed and 100 μL of 1% (w/v) bovine serum albumin, acting as a blocking agent, was added. After a 1-h incubation at room temperature, wells were washed three times with phosphate-buffered saline (PBS; 137 m*M* NaCl, 1.47 m*M* KH_2PO_4, 8.10 m*M* Na_2HPO_4, and 2.68 m*M* KCl, pH 7.4) containing 0.05% (v/v) Tween-20. Peroxidase samples were added to the wells and incubated at room temperature for 1 h. Wells were washed three times with PBS-Tween-20. A soluble, peroxidase chromogenic substrate (100 μL, tetramethylbenzadine) was added to the bound peroxidase. After 30 seconds, the reactions were stopped by the addition of 50 μL of 1N H_2SO_4 and three wells per replication were read at 450 nm (Fig. 2).

Example 4

Guaiacol Method

Purified peroxidase or seed coats were incubated in micro centrifuge tubes containing 1 ml of 0.5% (v/v) guaiacol at room temperature for 10 minutes before the addition of 50 μL of 0.1% (v/v) hydrogen peroxide. After 5 minutes, peroxidase activity was noted, with a brown solution being positive and a clear solution being negative. Peroxidase activity using a guaiacol substrate was also measured at 470 nm as described in Buttery and Buzzell, Crop Science, 8:722-725 (1968). Measurement of known peroxidase solutions, shows this procedure does not give a linear response and is therefore not suitable for plant breeding (Fig. 3).

Example 5

Method Comparison

In the ELISA procedure, we were unable to detect peroxidase with the 1:1000 and 1:5000 dilutions and the 1:100 dilution gave inconsistent results. Using the 1:10 dilution, we were able reproducibly to detect peroxidase. There was no increase in the optical density (OD) beyond 60 ng of peroxidase (Fig. 1).

In the PCA test, the 1:10000 dilution gave inconsistent results. Since the other dilutions gave similar results, the 1:5000 dilution was chosen because it uses the least amount of MAB (Fig. 2). Analysis of variance showed that a linear model explained the data ($R^2 = 0.99$).

Using a guaiacol substrate, peroxidase activity was measured at 470 nm (Fig. 3). Using analysis of variance, a linear model was inadequate to explain the data $R^2 = 0.77$).

ELISA and PCA Comparison

Boiled and nonboiled samples of purified peroxidase, were analyzed using both the ELISA and PCA assays. Presence or absence of peroxidase activities were checked using the guaiacol method (Buttery and Buzzell, 1968) (Table 1).

Analysis of Solutions With Known Peroxidase Activity

To determine if PCA could detect differences between samples with different peroxidase activities, samples with 100, 300, 390, 650, 670, 1500, and 2000 PPU/ml were analyzed using PCA (Fig. 4). There was no increase in the OD of the 1500 and 2000 PPU/ml samples over the 670 PPU/ml sample.

There was a major difference between what the PCA and ELISA techniques measured. The ELISA measures peroxidase concentration and not activity; the PCA measures activity not concentration. This was confirmed using the ELISA, PCA, and guaiacol procedures on boiled and nonboiled peroxidase samples. Comparison of the boiled and nonboiled OD of the guaiacol results obviously show the difference (Table 1). The guaiacol method showed high peroxidase activity in the nonboiled sample and no peroxidase activity in the boiled sample. The ELISA technique generated OD readings for both the boiled and nonboiled samples. There was a decrease in the ELISA OD between the boiled and nonboiled, which was probably attributable to destruction of the protein during the extended boiling of the sample. By comparison, the PCA OD was 0.0 in the boiled sample and 1.154 in the nonboiled sample. This is consistent with what one would expect looking at the differences between procedures. The ELISA technique used was a two-step indirect method. Conversely, in the PCA

technique, peroxidase was captured by the peroxidase monoclonal antibody coating the sample well. There was no secondary enzyme-linked antibody in the reaction. The peroxidase chromogen was added directly to the bound peroxidase, which reacted with the chromogen. Therefore, the PCA technique measures activity and not peroxidase concentration. This is why the boiled sample, which had no activity, had no PCA OD reading. Since the antibody captured peroxidase maintains enzymatic activity, the antibody must bind to an epitope not involved with enzymatic activity.

Solutions with known differences in peroxidase activity were analyzed to confirm the result that PCA gives a quantitative measure of peroxidase activity. Results show that the PCA can detect differences in solutions containing various levels of known peroxidase activity (Fig. 4).

Peroxidase activity also may be measured using guaiacol as a substrate. Comparison of the peroxidase activity curves clearly showed a difference between this method and PCA. There was a linear relationship using PCA, but a linear model was not adequate to describe the relationship using the guaiacol method. A higher order model was needed to explain the guaiacol curve. We believe the PCA technique was superior since the relationship may be explained by a simpler model.

Example 6

cDNA Library Construction

Total RNA was extracted from soybean (*Glycine max* cul. Resnik) seedbuds 21 days after flowering as previously described (20). Poly(A)-enriched RNA was prepared from total RNA using PolyATract and the cDNA library was constructed in the unidirectional vector Uni-ZAP XR.

Library Screening

A plant peroxidase specific primer (PSP) was generated from a conserved amino acid region (distal heme ligand, HFHDCFV, SEQ ID NO 1) in all plant peroxidases (5'CA(C/T)TT(T/C)CA(C/T)GA(C/T)TG(C/T)TT(C/T)GT3')(SEQ ID NO 2). The probe was generated using the 3'RACE system with soybean seedbud total RNA and PSP as described by the manufacture except that hot-start PCR was

performed. The PCR-RACE products were cloned into pCR™II plasmid. DNA from twenty clones was purified and digested with *Eco*R I, fractionated by electrophoresis on a 1% agarose gel, and blotted on a nylon membrane that was probed with [γ-^{32}p]dATP-end-labeled PSP. A single positive clone was random prime labeled with [α-^{32}p]dCTP and used for primary screening of the cDNA library (2.5 x 10^5 PFU). Prehybridization was conducted in 6x SSPE, 5x Denhardt's, 0.5% (w/v) SDS, 100μg/ml denatured salmon sperm DNA, and 50% formamide at 42°C for two hours. Hybridizations were performed overnight and the conditions were the same as those in prehybridization except that 1x Denhardt's was used.

PCR using PSP and the T7 vector primer flanking the cloning site was used to purify single phage clones. Phage particles were eluted by incubating primary picks and/or single plagues in 500 μl of SM buffer (SM: 100 mM NaCl, 10 mM $MgSO_4$, 0.01% w/v gelatin in 50 mM Tris pH 7.5) at room temperature for 2 hours. The PCR cycling parameters were 94°C, 1 minute at 57°C, and 1 minute at 72°C, and followed by a final extension at 72°C for 5 minutes. PCR reaction conditions were 1x reaction buffer (500 mM KCl, 100mM Tris-HCl, pH 9.0, 1.0% Triton X-100), 1.5 mM $MgCl_2$, 200 μM each dNTPs, one unit of *Taq* DNA polymerase, 1μM each primer and 2 μL of phage particle elution in 50 μL total.

DNA Sequencing and Sequence Analysis

DNA sequencing of both strands was performed using Sequenase Kit 2.0 (USB) and SK and KS primers (Stratagene). Synthetic primers corresponding to internal sequences of cDNA were made to complete sequencing. Sequence data were analyzed using GCG software (Madison, WI).

Example 7

Northern Blot Analysis and RT-PCR

Twenty-five μg of total RNA from various tissues were fractionated on 1% agarose gel containing formaldehyde, blotted onto nylon membrane, and probed with ^{32}P labeled probe. Both prehybridization and hybridization conditions were the same

as those described in library screening. Sample isolations and hybridizations were replicated twice.

cDNA specific primers designed from 3' untranslated regions of each cDNA and PSP were used in reverse transcript PCR (RT-PCR) to study expression patters. For *SEP*a1 (SEQ ID NO 10), *SEP*a2 (SEQ ID NO 12), *SEP*b1 (SEQ ID NO 14), and *SEP*b2 (SEQ ID NO 16) the primers were

5'AAATTAACTCAGCTGTGGG3' SEQ ID NO 3,

5'GGAACCCACTTATTCCATCG3' SEQ ID NO 4,

5'CCCAAGACATGCTTGAGAT3' SEQ ID NO 5, and

5'AAGTTCATACTTCTAAC3' SEQ ID NO 6, respectively.

Two μg of total RNA from different tissues of soybean were used for synthesizing the first strand of cDNA using SUPERSCRIPT™II Rnase H REVERSE TRANSCRIPTASE as suggested by the manufacture (BRL). RT-PCR conditions were the same as those in 3'RACE except that the annealing temperature for *SEP*b2 was 45°C.

Example 8

Isolation of Soybean Peroxidase cDNAs

The conserved amino acid sequence of plant peroxidases enabled the generation of molecular probe for plant peroxidase genes using 3'RACE. The 3'RACE experiment with PSP and adaptor primer complimentary to the oligo-d(T) end of the cDNA resulted in amplification of a 900-bp DNA fragment (data not shown). Using the fragment as probe, 25 clones were obtained by primary hybridization screening. Eleven positive clones were recovered after two rounds of PCR using PSP and T7 vector primers, and four clones, designated *SEP*a1, *SEP*a2, *SEP*b1, and *SEP*b2, were further analyzed.

Sequence Analysis of the cDNAs

The nucleotide sequences of the coding regions of *SEP*a1, *SEP*a2, *SEP*b1, and *SEP*b2, and their predicted amino acid sequences of their protein products, i.e., SEQ ID NOS 11, 13, 15, and 17, are shown in Figures 5 and 6. The coding regions of

*SEP*a1 and *SEP*a2 exhibit 97% amino acid identity, the coding regions of *SEP*b1 and *SEP*b2 have 95% amino acid identity, and the coding regions of *SEP*a1 and *SEP*b1 share 47% amino acid identity. Comparison of 168 bp, 3' untranslated regions of *SEP*a1 and *SEP*a2 revealed 83% homology. The homology between the 187 bp, 3' untranslated regions of *SEP*b1 and *SEP*b2 was 75%. There are 6 putative glycosylation sites specified by N-X-T/S at amino acid residues 56, 69, 128, 142, 183 and 214 in *SEP*a1 and *SEP*a2, and there are 4 putative glycosylation sites at residues 70, 142, 185 and 195 in *SEP*b1 and *SEP*b2, respectively; and *SEP*a1 and *SEP*a2 had the [Q L X X X F Y] SEQ ID NO 7 motif, where X is any amino acid, at the NH_2 terminus which is a feature found in most plant peroxidases. No [Q L X X X F Y] SEQ ID NO 7, motif exists in *SEP*b1 and *SEP*b2. Based on predicted amino acid sequences, all four proteins contain a predominantly hydrophobic amino acid signal sequences. Two copies of the putative polyadenylation signals AATAAG, SEQ ID NO 8 are present 39 and 106 bases upstream of the poly (A) signal in *SEP*a1 and 19 and 75 bases upstream in *SEP*a2. There is only one copy of the putative polyadenylation signal AATAAA 36 bases upstream of the poly (A) in *SEP*b1 and 14 bases upstream in *SEP*b2.

Example 9

Comparisons With Other Plant Peroxidase Sequences

Comparison between the predicted amino acid sequences of soybean peroxidases and some other plant peroxidase sequences. The levels of identity suggests that the clones encode peroxidases. There are three most highly conserved amino acid regions in almost all plant peroxidases. The first is from amino acid residues 33-55 with a predicted disulfide bridge in the middle and a potential heme binding site which belongs to a subdomain of 100% homology: HFHDCFV, SEQ ID NO 9. The second is from amino acid residues 89-105, again with two cysteines that may form disulfide bridges. The third is from amino acid residues 159-170 with a potential heme binding site in the middle. All of the peroxidases studied, except *SEP*b2, have eight cysteine residues that are located in similar positions in the primary

sequences, and two invariable histidine residues (at positions 42 and 167 in soybean peroxidases, Figure 5 and 6) are inferred in the active-site structure. The number of glycosylation sites vary greatly according to the isozymes (from 1 in peanut PNC2, 3 and 6 in soybean, to 8 in horseradish).

Differential Expressions of Peroxidase mRNAs

Total RNA from leaf, stem, root, seedbud, and developing seed were probed with a 300bp *Kpn-TifI* fragment from the 3' untranslated region of *SEP*a1. Data reveals that transcripts of approximately 1400 nucleotides from *SEP*a1 are present in developing seed and root. Since both the coding regions and the noncoding regions of the four cDNAs are high homologous, RT-PCR experiments were conducted to study the differential expressions of peroxidase mRNA. Data shows the amplification of cDNA synthesized from total RNA of different tissues with PSP and *SEP*a1-specific primer. To confirm the identity of RT-PCR products, RT-PCR products were transferred to nylon membrane and hybridized with *SEP*a1 from which *SEP*a1-specific primer was designed. Based on the results of RT-PCR with cDNA-specific primers, transcripts from *SEP*a2 were also detected in root and developing seed, and transcripts from *SEP*b1 and *SEP*b2 were detected in root, stem, leaf, and seedpod.

Example 10

Peroxidase Cloning

Our results demonstrate that PCR coupled with one round of conventional plaque lift hybridization was effective and rapid in both characterizing and screening of cDNA libraries provided that sequence information is available. This method would be especially useful when high density plating is used to obtain low abundance clones. Using PSP coupled with a vector primer, one can easily find the primary picks that are true positive clones. By replating the primary picks at low density, individual positive clones can be easily recovered by a second round of PCR with the same pair of primers. Directly using phage particle elution as template in PCR reactions without further precipitation was easily accomplished. The technique amplified a single, distinct product band from as few as 1 x 10^6 phage particles that

corresponds to ~0.1 ng of DNA, or as many as 1 x 10^8 phage particles have been used under the same amplification conditions with no detectable loss of specificity. Another advantage of this method is the size of the insert of positive clones can be predicted. A gene-specific primer coupled with vector primer also can be used to reveal the presence of genes of interest in a library prior to screening due to the high sensitivity of PCR. Failure to amplify any product of interest from the library may indicate that full-length cDNA of interest is not likely to be present in the library. In such case, unproductive screening can be avoided.

The predicted amino acid sequences of the four cDNA exhibit homology to other plant peroxidases indicating that the clones encode peroxidase. Each enzyme, except *SEP*b2, has eight cysteines in nearly identical positions in the primary sequences. Similar cysteines in horseradish and turnip enzymes had been shown to be involved in intramolecular disulfide linkages. By analogy with horseradish and turnip sequences four intrachain disulfide linkages can be predicted in the soybean isoperoxidases *SEP*a1 and *SEP*a2 (cysteine pairs between residues 11/89, 44/49/, 95/298 and 174/207).

There are three highly conserved amino acid sequences in all plant peroxidases. The first and the third contain the distal and proximal histidine residues concerned with binding the heme group. The first critical histidine ligand in *SEP*a1, *SEP*a2, *SEP*b1, and *SEP*b2 occurs at amino acid 42 in the mature proteins, thought to act in acid/base catalysis, and the second at 167 thought to bind the 5th ligand of heme iron. His-42 and His-167 are almost at identical positions in all plant peroxidases.

Plant peroxidases differ greatly in the number and the position of putative glycosylation sites and the heterogeneity of glycosylation indicated that peroxidases exist in differently glycosylated forms or glycoforms. Variability in *N*-linked oligosaccharide chain location may be adaptively important for fine tuning catalytic properties of the functional enzyme molecule. However, a glycosylation site at

residue 183 in *SEP*a1 and *SEP*a2 (185 in *SEP*b1 and *SEP*b2) is common to most plant peroxidases.

It is predicted from the cDNA sequences that all four proteins are initially synthesized as preproteins with predominantly hydrophobic amino acid signal sequences, suggesting that the mature proteins could be secreted through cell membranes. The hydrophobic residues in the signal peptides are of great importance and signal peptides are believed to function primarily by interacting favorably with the nonopolar interior of the membrane, entering and spanning it. All cloned plant peroxidases so far have a signal peptide and are therefor targeted to the secondary pathway. This was confirmed by biochemical studies of tobacco peroxidases localizing the peroxidases with pI 7.2-7.5 to the vacuoles and acidic peroxidases to the cell walls. It was reported that a C-terminal propeptide of 15 residues was necessary for proper sorting of barley lectin to vacuoles and that the vacuolar protein had this signal removed. Comparison of horseradish C protein and the cDNA derived sequences showed that 15 residues were removed at the C-terminus. The deduced amino acid sequences of soybean peroxidases showed no C-terminal extension present in peroxidases targeted to the vacuole.

Soybean peroxidases *SEP*b1 and *SEP*b2 may represent a new family of plant peroxidases and, perhaps, a new, unique biological function, as it is less than 50% amino acid identical to other known peroxidases. Cluster analysis of 2 plant peroxidases showed that *SEP*b1 and *SEP*b2 form a distinct group. *SEP*a1 and *SEP*a2 show about 67% amino acid identity to tomato anionic peroxidases *tap1* and *tap2*. Using *tap1* or *tap2* promoter/GUS fusions, the indution of the peroxidase genes by wounding and pathogen attack has been reported, (Mohan, et al., Plant Molecular Biology 21:341-354, 1993). This suggests a role of these peroxidase genes in wound healing process and in the plant defense response. A root-specific peroxidase gene has been described in *Nicotiana sylvestris* and its expression was initially linked to the initiation of the cell cycle of *in vitro* cultured protoplasts. Acidic tobacco peroxidase TOP A is a constitutive, cell wall bound peroxidase most abundant in root and stem

and thought to participate in secondary cell wall thickening. Over-expression of TOP A in transgenic tobacco gave rise to light-dependent wilting. A powdery mildew induced peroxidase pPOX381 of wheat leaves is about 90% identical to a constitutive wheat root peroxidase. The pPOX381 is 57% identical to TP 7, a highly basic peroxidase of the evolutionarily remote turnip, suggesting that these peroxidases might share common functional roles. These very different characteristics of plant peroxidase families may indicate that peroxidases have evolved to participate in very different biological functions.

Our results showed that RT-PCR with gene-specific primers is an effective and sensitive way to study expression of highly homologous genes. The result of RT-PCR was the same as that of Northern blotting, but RT-PCR in which 2 μg of total RNA was used is more sensitive than Northern blot in which 25 μg of total RNA was used in detection of gene expression. The expression patterns of the genes obtained from both northern analysis and RT-PCR indicates differential expressions of various genes. In studies of other plants, there was evidence of differential expression of peroxidase genes. It is not apparent why some organisms have a relatively large number of expressed peroxidase genes. One possibility is that the different encoded proteins have different functions. However, different isoforms can be produced by post-translational modification, suggesting that different genes might not be necessary to provide different functions. A second possibility is that multiple genes could allow for greater regulatory flexibility. Some genes may be expressed in specific organs or at specific stages, and the expression of the genes may be determined by different signals. Regulations studies of the different peroxidase genes and the specific functions of their products are under way.

Example 11

Detection of Soybean Cyst Nematode Feeding

Soybean cyst nematode (SCN) is a major pest of soybean, which decreases yield by feeding on roots. Seedlings from 4 SCN resistant and 2 susceptible cultivars were challenged with 3000 SCN juveniles. Control seedlings were not challenged

with SCN. Samples were collected at 0, 1, 2, 3 and 4 weeks and peroxidase activity assayed according to example 3. There was no increase in peroxidase activity at weeks 1 and 2. There was increased peroxidase activity in all cultivars at week 3 (range 3 to 89%). At week 4 the increase in activity ranged from 4 to 41%. By week 5 there was no increased peroxidase activity in the SCN challenged samples. Samples were taken from root tissue.

Example 12

Quantitation of Peroxidase Activity in Stored Seeds

Seeds from high peroxidase soybean cultivars were stored under various conditions to determine factors that affect peroxidase activity. Two replicates of seed lots were stored at 10°C, 20°C, 30°C, 40°C and warehouse conditions. Seed were equilibrated to moistures of 9 and 13%. Samples were drawn monthly except for 40°C, which was drawn weekly. Peroxidase activity was determined according to Example 3. Results show that the greater the temperature, the greater the decrease in peroxidase activity.

Example 13

Immunopurification of Peroxidase

Peroxidase was purified from plant fluid and solutions by immunoprecipitation. Solutions containing peroxidase were mixed with said antibody. Protein A-Sepharose was added to the peroxidase/antibody mixture and incubated for one hour at 4°C. The tertiary protein A - peroxidase antibody complex was collected by centrifugation and washed three times. The resuspended sepharose beads were incubated at 4°C for 20 minutes. After the last wash, 30 μl of gel-loading buffer was added to the beads. Samples were heated to 100°C for 3 minutes and the protein A-sepharose was removed by centrifugation. Purified proteins were separated on a nondenaturing acrylamide gel and visualized by histochemical staining using tetramethylbenzadine as a chromogen. Results shaved a single peroxidase band on the gel.

Example 14

Crop and Cultivar Screening

The use of said antibody is not limited to soybean. In soybeans though, 306 plant introductions from USDA and 33 cultivars were screened for peroxidase activity (Fig. 7). The invention is also useful for screening segregating populations as in a plant breeding program. The means from three replications of the high-peroxidase cultivars used as parents in the cross, Winchester and Resnik, were 0.502± 0.038 and 0.777± 0.082 respectively. PCA detected differences in a segregating population (Fig. 8). One hundred fifteen progeny from a cross of two high peroxidase cultivars were screened for peroxidase activity. Genotypes with peroxidase activity higher than both parents were identified. The said invention also detected differences in peroxidase activity between 9 sorghum, 5 wheat, 5 corn and 2 oat cultivars.

Analysis of the segregating population showed that PCA can detect differences in peroxidase activity and genotypes with activity greater than the highest parent were identified. PCA will therefore be useful in the introgression of high peroxidase activity into breeding lines. The PCA technique uses the same equipment as the ELISA technique and large scale screening will therefore be routinely available. Results show that peroxidase can be easily extracted from seed coats without destroying the seed. Besides being a valuable procedure for screening cultivars for high peroxidase activity, this technique also will permit investigations of the effect environment and seed storage have on peroxidase activity.

Example 15

Increased Peroxidase Activity in Plants

Peroxidase activity can be increased through plant breeding as described in Example 14. Another method is through plant transformation. Duplicate copies of the gene may be incorporated into plants. Another manifestation is the transformation of altered or mutant copies of the gene. DNA sequences may be altered by means of in vitro mutagenesis and alteration of the regulatory regions, promoter, 5'- and 3' untranslated regions, coding regions or termination sequences may increase expression

of the peroxidase gene. Transformation and production of peroxidase is not limited to soybeans and may be accomplished in plants that are transformable.

Example 16

Production of Peroxidase in Bacteria

A single recombinant colony was incubated overnight at 37°C in 3 ml of LB medium containing 100 μg/ml ampicillin. One ml of culture was used to inoculate 50 ml of fresh LB containing ampicillin and allowed to grow to an OD_{600}=0.5. IPTG was added to a final concentration of 0.5 mM and incubated for an additional 4 hours. Two hundred μl of the culture was pelleted by centrifugation and resuspended in 100 μl of TE. Bacteria was homogenized for 45 seconds with an acetal pestle. The homogenate was centrifuged and 50 μl of the supernatant was analyzed on both an acrylamide gel and the invention as stated in example 3. Functional peroxidase was isolated from bacterial cultures.

Example 17

Genomic Library Construction and Screening

Soybean nuclear DNA was restriction digested with Xho I and ligated into Xho I digested EMBL3 SP6/T7 lambda arms (Stratagene). The genomic library was screened by one round of lift hybridization and positive clones were purified by two rounds of PCR screening. For lift hybridizations, 5 x 10^5 plaques were plated and hybridized with a mixture of ^{32}P-dCTP randomly labeled cDNAs from example 6. Two rounds of PCR screening were performed on 14 clones to purify positive clones. PCR primers designed from 5' and 3' ultratranslated regions of the 4 cDNAs (examples 6 and 8) were used in PCR screening. Four genomic clones were recovered.

Example 18

Production of Transgenes in Soybean

Transformed plants comprising a recombinant DNA sequence under modified or unmodified transcriptional and translational control of the peroxidase promoter and containing the hydrophobic leader sequence and a sequence encoding a protein or

polypeptide will be expressed in the seed coat. Expressed transgenes may be antigenic and act as an animal or human vaccine. Transgenes also may be enzymes or nonenzymatic proteins.

Example 19

Solid-Phase Peroxidase

Peroxidase captured by the said antibody still maintains oxidative activity, therefore antibody bound peroxidase can be immobilized on a solid state matrix (e.g. polystyrene, sepharose column). In oxidative reactions where peroxidase is being used, reagents may be passed through or over immobilized peroxidase and product or modified reagents collected.

Example 20

Non-radioactive Detection of Nucleic Acids

Peroxidase can be covalently conjugated to oligonucleotides. This conjugate can be used as a probe in hybridization assays and in polymerase chain reaction procedures as described in Patents 5,254,469 and 5,272,077. The said antibody can be used to purify the oligonucleotide peroxidase conjugate (Example 13). Said antibody may be conjugated with enzyme, such as peroxidase, glucose oxidase, alkaline phosphatase and beta-galactosidase and used in the detection of nucleic acid providing an appropriate chromogen, fluorogen, chemiluminescent or substrate is provided.

While the invention has been disclosed in this patent application by reference to the details of the preferred embodiments of the invention, it is to be understood that this disclosure is intended in an illustrative rather than a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

Table 1. Comparison of boiled and nonboiled peroxidase samples.

	Assays		
Peroxidase	ELISA[1]	SPCA[2]	Guaiacol[3]
	Absorbance		
Nonboiled	1.007	1.154	+
Boiled	0.806	0.000	-

[1] 405 nm.

[2] 450 nm.

[3] +, activity; - ,no activity.

Table 2. Percentage of similarity and identity at amino acid level among the mature proteins encoded by *SEP*a1, *SEP*a2, *SEP*b1 and *SEP*b2 and different plant peroxidases.

	Similarity		Identity	
	*SEP*a1/*SEP*a2	*SEP*b1/*SEP*b2	*SEP*a1/*SEP*a2	*SEP*b1/*SEP*b2
Tomato	78	59	67	43
Barley	66	63	46	42
Wheat	58	59	40	40
Horseradish	60	58	46	42
Peanut	58	58	43	40
Turnip	55	64	41	44
Tobacco	57	58	40	39
Cucumber	60	59	44	42
Arabidopsis	58	56	41	40

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: INDIANA CROP IMPROVEMENT ASSOCIATION
(B) STREET: 3510 U.S. 52 SOUTH
(C) CITY: LAFAYETTE
(D) STATE: INDIANA
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 47905
(G) TELEPHONE:
(H) TELEFAX:

(ii) TITLE OF INVENTION: A SOYBEAN PEROXDIASE GENE FAMILY AND AN ASSAY FOR DETECTING SOYBEAN PEROXIDASE ACTIVITY

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/549,658
(B) FILING DATE: 27-OCT-1995

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

His Phe His Asp Cys Phe Val
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3
(D) OTHER INFORMATION: /note= "Location 3 can be either C or T"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 6
(D) OTHER INFORMATION: /note= "Location 6 can be either T or C"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "Location 9 can be either C or T"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 12
(D) OTHER INFORMATION: /note= "Location 12 can be either C or T"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 15
(D) OTHER INFORMATION: /note= "Location 15 can be either C or T"

(ix) FEATURE:
(A) NAME/KEY: misc_feature

(B) LOCATION: 18
(D) OTHER INFORMATION: /note= "Location 18 can be either C or T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CAYTTYCAYG AYTGYTTYGT 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAATTAACTC AGCTGTGGG 19

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGAACCCACT TATTCCATCG 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCCAAGACAT GCTTGAGAT 19

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucl ic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGTTCATAC TTCTAAC 17

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gln Leu Xaa Xaa Xaa Phe Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala Ala Thr Ala Ala Ala
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

His Phe His Asp Cys Phe Val
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1315 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: 5'UTR
(B) LOCATION: 1..82

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 83..1054

(ix) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 1055..1315

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 83..145

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 146..1054

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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GATTTCTGCC TGCTGCATCA AA ATG GGA AGC AAC TTG AGG TTT TTG AGT CTT       112
                         Met Gly Ser Asn Leu Arg Phe Leu Ser Leu
                         -21 -20                 -15

TGC CTC TTG GCA TTG ATT GCA TCG ACT CAT GCT CAA CTT CAG CTT GGT       160
Cys Leu Leu Ala Leu Ile Ala Ser Thr His Ala Gln Leu Gln Leu Gly
    -10                 -5                  1               5

TTT TAT GCT AAC AGT TGC CCA AAA GCA GAG CAA ATT GTT TTG AAA TTT       208
Phe Tyr Ala Asn Ser Cys Pro Lys Ala Glu Gln Ile Val Leu Lys Phe
                 10                  15                  20

GTT CAT GAC CAT ATC CAC AAT GCT CCA TCA CTA GCA GCT GCA TTA ATA       256
Val His Asp His Ile His Asn Ala Pro Ser Leu Ala Ala Ala Leu Ile
            25                  30                  35

AGA ATG CAC TTT CAT GAC TGT TTT GTA AGG GGA TGT GAT GCA TCA GTC       304
Arg Met His Phe His Asp Cys Phe Val Arg Gly Cys Asp Ala Ser Val
        40                  45                  50

CTT CTG AAC TCA ACA ACC AAT CAG GCT GAG AAG AAT GCT CCT CCA AAT       352
Leu Leu Asn Ser Thr Thr Asn Gln Ala Glu Lys Asn Ala Pro Pro Asn
    55                  60                  65
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CTC ACA GTA AGA GGC TTT GAC TTC ATT GAC AGA ATA AAG AGC CTT GTT        400
Leu Thr Val Arg Gly Phe Asp Phe Ile Asp Arg Ile Lys Ser Leu Val
 70                  75                  80                  85

GAA GCT GAA TGC CCT GGT GTG GTC TCT TGT GCT GAT ATC CTC ACT TTG        448
Glu Ala Glu Cys Pro Gly Val Val Ser Cys Ala Asp Ile Leu Thr Leu
                 90                  95                 100

GCT GCC AGA GAC ACT ATT GTA GCC ACA GGT GGA CCT TTT TGG AAA GTT        496
Ala Ala Arg Asp Thr Ile Val Ala Thr Gly Gly Pro Phe Trp Lys Val
            105                 110                 115

CCA ACT GGT CGA AGG GAT GGG GTC GTC TCT AAC TTG ACG GAA GCC AGA        544
Pro Thr Gly Arg Arg Asp Gly Val Val Ser Asn Leu Thr Glu Ala Arg
        120                 125                 130

AAT AAC ATT CCT GCT CCA TCT TCC AAC TTT ACC ACC CTA CAA ACA CTC        592
Asn Asn Ile Pro Ala Pro Ser Ser Asn Phe Thr Thr Leu Gln Thr Leu
    135                 140                 145

TTT GCT AAC CAA GGA CTT GAT TTG AAG GAC TTG GTC CTG CTC TCT GGT        640
Phe Ala Asn Gln Gly Leu Asp Leu Lys Asp Leu Val Leu Leu Ser Gly
150                 155                 160                 165

GCT CAC ACA ATT GGT ATC GCT CAT TGC TCA TCA TTA TCA AAC CGG TTG        688
Ala His Thr Ile Gly Ile Ala His Cys Ser Ser Leu Ser Asn Arg Leu
                170                 175                 180

TTC AAT TTC ACT GGC AAG GGT GAT CAA GAC CCG TCA CTA GAT AGT GAA        736
Phe Asn Phe Thr Gly Lys Gly Asp Gln Asp Pro Ser Leu Asp Ser Glu
            185                 190                 195

TAT GCT GCA AAT TTG AAA GCA TTC AAG TGC ACA GAC CTC AAC AAG TTG        784
Tyr Ala Ala Asn Leu Lys Ala Phe Lys Cys Thr Asp Leu Asn Lys Leu
        200                 205                 210


AAC ACC ACA AAA ATT GAG ATG GAC CCT GGA AGT CGC AAG ACA TTT GAT        832
Asn Thr Thr Lys Ile Glu Met Asp Pro Gly Ser Arg Lys Thr Phe Asp
    215                 220                 225

CTT AGC TAC TAT AGT CAC GTT ATT AAG AGA AGG GGT CTA TTT GAG TCA        880
Leu Ser Tyr Tyr Ser His Val Ile Lys Arg Arg Gly Leu Phe Glu Ser
230                 235                 240                 245

GAT GCT GCA TTA TTG ACT AAC TCA GTT ACA AAG GCA CAA ATC ATC CAA        928
Asp Ala Ala Leu Leu Thr Asn Ser Val Thr Lys Ala Gln Ile Ile Gln
                250                 255                 260

TTG CTT GAA GGG TCA GTT GAA AAT TTC TTT GCT GAG TTT GCA ACC TCC        976
Leu Leu Glu Gly Ser Val Glu Asn Phe Phe Ala Glu Phe Ala Thr Ser
            265                 270                 275

ATC GAG AAA ATG GGA AGA ATT AAT GTG AAG ACA GGC ACA GAA GGA GAG       1024
Ile Glu Lys Met Gly Arg Ile Asn Val Lys Thr Gly Thr Glu Gly Glu
        280                 285                 290
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Ile Arg Lys His Cys Ala Phe Ile Asn Ser
    295                 300

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CTACATACGT GTGATCCTTT ATGGTATGGT TGTTGTATGT GTGTTGGAAT AAGTGGGCTC     1194

TTAAGTTATT CATATTTCCA ACTTTCCAAC TTTGCTGGTA GATCATGCTC TTGTAATAAG     1254

AACCAGAATT TTTTGTGCTA CCCACAGCTG AGTTAATTTA AAAAAAAAAA AAAAAAAAAA     1314

A                                                                     1315
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(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 324 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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 -5                  1                   5                  10


Pro Lys Ala Glu Gln Ile Val Leu Lys Phe Val His Asp His Ile His
             15                  20                  25

Asn Ala Pro Ser Leu Ala Ala Ala Leu Ile Arg Met His Phe His Asp
         30                  35                  40

Cys Phe Val Arg Gly Cys Asp Ala Ser Val Leu Leu Asn Ser Thr Thr
     45                  50                  55

Asn Gln Ala Glu Lys Asn Ala Pro Pro Asn Leu Thr Val Arg Gly Phe
 60                  65                  70                  75

Asp Phe Ile Asp Arg Ile Lys Ser Leu Val Glu Ala Glu Cys Pro Gly
                 80                  85                  90

Val Val Ser Cys Ala Asp Ile Leu Thr Leu Ala Ala Arg Asp Thr Ile
             95                 100                 105

Val Ala Thr Gly Gly Pro Phe Trp Lys Val Pro Thr Gly Arg Arg Asp
        110                 115                 120

Gly Val Val Ser Asn Leu Thr Glu Ala Arg Asn Asn Ile Pro Ala Pro
    125                 130                 135
```

```
Ser Ser Asn Phe Thr Thr Leu Gln Thr Leu Phe Ala Asn Gln Gly Leu
140                 145                 150                 155

Asp Leu Lys Asp Leu Val Leu Leu Ser Gly Ala His Thr Ile Gly Ile
                160                 165                 170

Ala His Cys Ser Ser Leu Ser Asn Arg Leu Phe Asn Phe Thr Gly Lys
            175                 180                 185

Gly Asp Gln Asp Pro Ser Leu Asp Ser Glu Tyr Ala Ala Asn Leu Lys
        190                 195                 200

Ala Phe Lys Cys Thr Asp Leu Asn Lys Leu Asn Thr Thr Lys Ile Glu
    205                 210                 215

Met Asp Pro Gly Ser Arg Lys Thr Phe Asp Leu Ser Tyr Tyr Ser His
220                 225                 230                 235

Val Ile Lys Arg Arg Gly Leu Phe Glu Ser Asp Ala Ala Leu Leu Thr
                240                 245                 250

Asn Ser Val Thr Lys Ala Gln Ile Ile Gln Leu Leu Glu Gly Ser Val
            255                 260                 265

Glu Asn Phe Phe Ala Glu Phe Ala Thr Ser Ile Glu Lys Met Gly Arg
        270                 275                 280

Ile Asn Val Lys Thr Gly Thr Glu Gly Glu Ile Arg Lys His Cys Ala
    285                 290                 295

Phe Ile Asn Ser
300
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1326 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: 5'UTR
(B) LOCATION: 1..86

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 87..1058

(ix) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 1059..1326

(ix) FEATURE:
(A) NAME/KEY: sig_peptide

(B) LOCATION: 87..149

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 150..1058

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
GCCTCTTTCA AGAAGCATCT GAGTGCTTAT TATTTGTAAT ATATATAGTC ACTCAAGCTT      60

CTAGGATTTG TGCCAGCTAC ATGAAA ATG GGA AGC AAC TTC AGG TTT TTG AGT      113
                             Met Gly Ser Asn Phe Arg Phe Leu Ser
                             -21 -20                 -15

CTT TGC CTC TTG GCA TTG ATT GCA TCA ACC CAT GCT CAA CTT CAG CTT       161
Leu Cys Leu Leu Ala Leu Ile Ala Ser Thr His Ala Gln Leu Gln Leu
        -10                  -5                  1

GGT TTT TAT GCC AAG AGT TGC CCA AAC GCT GAG CAA ATC GTT TTG AAA       209
Gly Phe Tyr Ala Lys Ser Cys Pro Asn Ala Glu Gln Ile Val Leu Lys
  5                  10                  15                  20

TTT GTC CAT GAC CAT ATC CAC AAT GCT CCA TCA CTA GCA GCT GCA TTG       257
Phe Val His Asp His Ile His Asn Ala Pro Ser Leu Ala Ala Ala Leu
                 25                  30                  35

ATA AGA ATG CAC TTC CAT GAC TGT TTT GTA AGG GGA TGT GAT GCA TCA       305
Ile Arg Met His Phe His Asp Cys Phe Val Arg Gly Cys Asp Ala Ser
             40                  45                  50

GTC CTT CTG AAC TCA ACA ACC AAT CAA GCT GAA AAG AAT GCT CCT CCA       353
Val Leu Leu Asn Ser Thr Thr Asn Gln Ala Glu Lys Asn Ala Pro Pro
         55                  60                  65

AAT CTC ACA GTA AGA GGC TTT GAC TTC ATT GAC AGA ATA AAG AGC CTT       401
Asn Leu Thr Val Arg Gly Phe Asp Phe Ile Asp Arg Ile Lys Ser Leu
     70                  75                  80

GTT GAG GCA GAA TGC CCT GGT GTG GTC TCT TGT GCT GAT ATC CTC ACT       449
Val Glu Ala Glu Cys Pro Gly Val Val Ser Cys Ala Asp Ile Leu Thr
 85                  90                  95                  100

TTG TCT GCC AGA GAC ACT ATT GTA GCC ACA GGT GGA CCA TTT TGG AAA       497
Leu Ser Ala Arg Asp Thr Ile Val Ala Thr Gly Gly Pro Phe Trp Lys
                105                 110                 115

GTT CCA ACA GGT CGA AGA GAT GGG GTC ATC TCT AAC TTG ACG GAA GCC       545
Val Pro Thr Gly Arg Arg Asp Gly Val Ile Ser Asn Leu Thr Glu Ala
            120                 125                 130

AGA GAT AAC ATT CCT GCT CCA TCT TCT AAC TTT ACC ACC CTA CAA ACA       593
Arg Asp Asn Ile Pro Ala Pro Ser Ser Asn Phe Thr Thr Leu Gln Thr
        135                 140                 145

CTC TTT GCC AAC CAA GGA CTT GAT TTG AAG GAC TTG GTC CTG CTC TCT       641
Leu Phe Ala Asn Gln Gly Leu Asp Leu Lys Asp Leu Val Leu Leu Ser
```

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    150                 155                 160

GGT GCT CAC ACA ATT GGT ATC GCT CAT TGC TCA TCA TTG TCA AAC CGC      689
Gly Ala His Thr Ile Gly Ile Ala His Cys Ser Ser Leu Ser Asn Arg
165                 170                 175                 180

TTG TTC AAT TTC ACT GGC AAG GGT GAT CAA GAC CCG TCA TTA GAC AGT      737
Leu Phe Asn Phe Thr Gly Lys Gly Asp Gln Asp Pro Ser Leu Asp Ser
                185                 190                 195

GAA TAT GCT GCA AAT CTG AAA GCC TTC AAG TGC ACG GAC CTC AAT AAG      785
Glu Tyr Ala Ala Asn Leu Lys Ala Phe Lys Cys Thr Asp Leu Asn Lys
            200                 205                 210

TTG AAC ACC ACA AAA ATT GAG ATG GAC CCT GGA AGT CGC AAG ACA TTT      833
Leu Asn Thr Thr Lys Ile Glu Met Asp Pro Gly Ser Arg Lys Thr Phe
        215                 220                 225

GAT CTT AGC TAC TAT AGT CAT GTG ATT AAG AGA AGG GGT CTA TTT GAG      881
Asp Leu Ser Tyr Tyr Ser His Val Ile Lys Arg Arg Gly Leu Phe Glu
    230                 235                 240

TCA GAT GCT GCA TTG TTG ACA AAC TCA GTT ACA AAG GCT CAA ATC ATT      929
Ser Asp Ala Ala Leu Leu Thr Asn Ser Val Thr Lys Ala Gln Ile Ile
245                 250                 255                 260

GAA TTG CTT GAA GGG TCA GTT GAA AAT TTC TTT GCT GAG TTT GCA ACC      977
Glu Leu Leu Glu Gly Ser Val Glu Asn Phe Phe Ala Glu Phe Ala Thr
                265                 270                 275

TCC ATG GAG AAA ATG GGA AGA ATT AAT GTA AAG ACA GGG ACA GAA GGA     1025
Ser Met Glu Lys Met Gly Arg Ile Asn Val Lys Thr Gly Thr Glu Gly
            280                 285                 290

GAG ATC AGG AAG CAT TGT GCA TTT CTA AAT AGC TAAGAATCTT GTCTTGTTCA   1078
Glu Ile Arg Lys His Cys Ala Phe Leu Asn Ser
        295                 300

TGGATGAATC TTGTATCATT TATTTTTTGG GTTTGGTTAT TTATGCTATG CCATGTTTTT   1138

TTATTAGTTA TGCTATGCCA TGTGGTGTCT GTCTACATAT GAGTGATCCC GTATGGTATG   1198

GTTGTTGTAT GTGCGATGGA ATAAGTGGGT TCCATTGTTA TTCTTATAAT TTCCAACTTT   1258

GCTGGTAGAT CTTGTAATAA GAAGCAGAAT TTCTTGTGCT AAAAAAAAAA AAAAAAAAAA   1318

AAAAAAAA                                                            1326
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 324 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Gly Ser Asn Phe Arg Ph  Leu Ser Leu Cys Leu Leu Ala Leu Ile
-21 -20                -15                 -10

Ala Ser Thr His Ala Gln Leu Gln Leu Gly Phe Tyr Ala Lys Ser Cys
 -5                  1                   5                  10

Pro Asn Ala Glu Gln Ile Val Leu Lys Phe Val His Asp His Ile His
             15                  20                  25

Asn Ala Pro Ser Leu Ala Ala Ala Leu Ile Arg Met His Phe His Asp
         30                  35                  40

Cys Phe Val Arg Gly Cys Asp Ala Ser Val Leu Leu Asn Ser Thr Thr
     45                  50                  55

Asn Gln Ala Glu Lys Asn Ala Pro Pro Asn Leu Thr Val Arg Gly Phe
 60                  65                  70                  75

Asp Phe Ile Asp Arg Ile Lys Ser Leu Val Glu Ala Glu Cys Pro Gly
                 80                  85                  90

Val Val Ser Cys Ala Asp Ile Leu Thr Leu Ser Ala Arg Asp Thr Ile
             95                 100                 105

Val Ala Thr Gly Gly Pro Phe Trp Lys Val Pro Thr Gly Arg Arg Asp
        110                 115                 120

Gly Val Ile Ser Asn Leu Thr Glu Ala Arg Asp Asn Ile Pro Ala Pro
    125                 130                 135

Ser Ser Asn Phe Thr Thr Leu Gln Thr Leu Phe Ala Asn Gln Gly Leu
140                 145                 150                 155

Asp Leu Lys Asp Leu Val Leu Leu Ser Gly Ala His Thr Ile Gly Ile
                160                 165                 170

Ala His Cys Ser Ser Leu Ser Asn Arg Leu Phe Asn Phe Thr Gly Lys
            175                 180                 185

Gly Asp Gln Asp Pro Ser Leu Asp Ser Glu Tyr Ala Ala Asn Leu Lys
        190                 195                 200

Ala Phe Lys Cys Thr Asp Leu Asn Lys Leu Asn Thr Thr Lys Ile Glu
    205                 210                 215

Met Asp Pro Gly Ser Arg Lys Thr Phe Asp Leu Ser Tyr Tyr Ser His
220                 225                 230                 235

Val Ile Lys Arg Arg Gly Leu Phe Glu Ser Asp Ala Ala Leu Leu Thr
                240                 245                 250

Asn Ser Val Thr Lys Ala Gln Ile Ile Glu Leu Leu Glu Gly Ser Val
            255                 260                 265

Glu Asn Phe Phe Ala Glu Phe Ala Thr Ser Met Glu Lys Met Gly Arg
```

```
        270                 275                 280

Ile Asn Val Lys Thr Gly Thr Glu Gly Glu Ile Arg Lys His Cys Ala
    285                 290                 295

Phe Leu Asn Ser
300
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1191 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: 5'UTR
(B) LOCATION: 1..59

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 60..998

(ix) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 999..1191

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 60..122

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 123..998

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
GGCACGAGGA GAGAGAGAGA GAGAGAACTA GTCTCGAGCA TCAAAGTACT CAAATTAGC        59

ATG GCT GTC ATG GTT GCA TTC TTG AAT TTG ATC ATC TTT TCA GTA GTC        107
Met Ala Val Met Val Ala Phe Leu Asn Leu Ile Ile Phe Ser Val Val
-21 -20                 -15                 -10

TCT ACA ACA GGC AAG TCA CTG AGC TTA AAC TAC TAT GCA AAA ACA TGC        155
Ser Thr Thr Gly Lys Ser Leu Ser Leu Asn Tyr Tyr Ala Lys Thr Cys
 -5                  1                   5                  10

CCT AAT GTG GAG TTC ATT GTT GCC AAG GCA GTA AAG GAT GCC ACT GCT        203
Pro Asn Val Glu Phe Ile Val Ala Lys Ala Val Lys Asp Ala Thr Ala
             15                  20                  25

AGG GAC AAA ACT GTT CCA GCA GCA ATT CTG CGA ATG CAC TTC CAT GAT        251
Arg Asp Lys Thr Val Pro Ala Ala Ile Leu Arg Met His Phe His Asp
         30                  35                  40
```

```
TGT TTC GTT CGG GGG TGT GAT GCC TCT GTG CTG CTA AAT TCA AAA GGA      299
Cys Phe Val Arg Gly Cys Asp Ala Ser Val Leu Leu Asn Ser Lys Gly
     45                  50                  55

AAC AAC AAA GCA GAA AAA GAC GGG CCA CCA AAT GTT TCT TTG CAT GCA      347
Asn Asn Lys Ala Glu Lys Asp Gly Pro Pro Asn Val Ser Leu His Ala
 60                  65                  70                  75

TTC TAT GTC ATT GTA GCA GCA AAG AAA GCA CTA GAA GCT TCA TGC CCT      395
Phe Tyr Val Ile Val Ala Ala Lys Lys Ala Leu Glu Ala Ser Cys Pro
                 80                  85                  90

GGT GTG GTC TCT TGT GCT GAC ATC CTT GCT CTG GCA GCA AGG GTC GCA      443
Gly Val Val Ser Cys Ala Asp Ile Leu Ala Leu Ala Ala Arg Val Ala
             95                 100                 105

GTT TTT CTG TCA GGA GGA CCT ACA TGG GAT GTT CCT AAA GGA AGA AAG      491
Val Phe Leu Ser Gly Gly Pro Thr Trp Asp Val Pro Lys Gly Arg Lys
        110                 115                 120

GAT GGT AGA ACA TCT AAA GCC AGT GAA ACC AGA CAA TTG CCA GCA CCA      539
Asp Gly Arg Thr Ser Lys Ala Ser Glu Thr Arg Gln Leu Pro Ala Pro
    125                 130                 135

ACC TTC AAC TTA TCA CAA CTG CGG CAA AGT TTC TCT CAA AGA GGA CTG      587
Thr Phe Asn Leu Ser Gln Leu Arg Gln Ser Phe Ser Gln Arg Gly Leu
140                 145                 150                 155

TCA GGG GAA GAC CTG GTA GCT CTG TCA GGG GGG CAC ACT TTG GGT TTC      635
Ser Gly Glu Asp Leu Val Ala Leu Ser Gly Gly His Thr Leu Gly Phe
                160                 165                 170

TCT CAC TGC TCA TCT TTC AAG AAC AGA ATC CAC AAC TTC AAT GCA ACA      683
Ser His Cys Ser Ser Phe Lys Asn Arg Ile His Asn Phe Asn Ala Thr
            175                 180                 185

CAT GAT GTT GAC CCT TCA TTA AAT CCA TCA TTT GCA GCA AAA CTG ATC      731
His Asp Val Asp Pro Ser Leu Asn Pro Ser Phe Ala Ala Lys Leu Ile
        190                 195                 200

TCA ATT TGT CCA CTA AAA AAT CAG GCA AAA AAT GCA GGC ACC TCT ATG      779
Ser Ile Cys Pro Leu Lys Asn Gln Ala Lys Asn Ala Gly Thr Ser Met
    205                 210                 215

GAC CCT TCA ACA ACA ACT TTT GAT AAT ACA TAT TAC AGG TTG ATC CTC      827
Asp Pro Ser Thr Thr Thr Phe Asp Asn Thr Tyr Tyr Arg Leu Ile Leu
220                 225                 230                 235

CAA CAG AAA GGC TTG TTT TCT TCT GAT CAA GTT TTG CTT GAC AAC CCA      875
Gln Gln Lys Gly Leu Phe Ser Ser Asp Gln Val Leu Leu Asp Asn Pro
                240                 245                 250

GAC ACT AAA AAT CTG GTT ACA AAG TTT GCC ACC TCA AAA AAG GCT TTT      923
Asp Thr Lys Asn Leu Val Thr Lys Phe Ala Thr Ser Lys Lys Ala Phe
            255                 260                 265

TAT GAG GCT TTT GCG AAG TCC ATG ATC AGA ATG AGT AGC TAC AAT GGT      971
```

```
Tyr Glu Ala Phe Ala Lys Ser Met Ile Arg Met Ser Ser Tyr Asn Gly
        270                 275                 280

GGA CAG GAG GTT AGA AGG ACT GCA GAA TGATCAATTA ATAAGTCTTA          1018
Gly Gln Glu Val Arg Arg Thr Ala Glu
    285                 290

AATCAATTCA AGTTAAATTG ATGTTCCAAA CAAGTTGGAT CAAATTTCCT AGATGCCAAG  1078

ATATTATGTC TTTTTCCTCT ATTAAAGAAA TATGTATATT TATCTGAAGT TAATAAAATC  1138

TCAAGCATGT CTTGGGAAAT TAATTTAGAG CTCAAAAAAA AAAAAAAAAA AAA          1191
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 313 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Ala Val Met Val Ala Phe Leu Asn Leu Ile Ile Phe Ser Val Val
-21 -20                 -15                 -10

Ser Thr Thr Gly Lys Ser Leu Ser Leu Asn Tyr Tyr Ala Lys Thr Cys
 -5                  1                   5                  10

Pro Asn Val Glu Phe Ile Val Ala Lys Ala Val Lys Asp Ala Thr Ala
             15                  20                  25

Arg Asp Lys Thr Val Pro Ala Ala Ile Leu Arg Met His Phe His Asp
         30                  35                  40

Cys Phe Val Arg Gly Cys Asp Ala Ser Val Leu Leu Asn Ser Lys Gly
     45                  50                  55

Asn Asn Lys Ala Glu Lys Asp Gly Pro Pro Asn Val Ser Leu His Ala
 60                  65                  70                  75

Phe Tyr Val Ile Val Ala Ala Lys Lys Ala Leu Glu Ala Ser Cys Pro
                 80                  85                  90

Gly Val Val Ser Cys Ala Asp Ile Leu Ala Leu Ala Ala Arg Val Ala
             95                 100                 105

Val Phe Leu Ser Gly Gly Pro Thr Trp Asp Val Pro Lys Gly Arg Lys
        110                 115                 120

Asp Gly Arg Thr Ser Lys Ala Ser Glu Thr Arg Gln Leu Pro Ala Pro
    125                 130                 135

Thr Phe Asn Leu Ser Gln Leu Arg Gln Ser Phe Ser Gln Arg Gly Leu
140                 145                 150                 155
```

```
Ser Gly Glu Asp Leu Val Ala Leu Ser Gly Gly His Thr Leu Gly Phe
                160                 165                 170

Ser His Cys Ser Ser Phe Lys Asn Arg Ile His Asn Phe Asn Ala Thr
            175                 180                 185

His Asp Val Asp Pro Ser Leu Asn Pro Ser Phe Ala Ala Lys Leu Ile
        190                 195                 200

Ser Ile Cys Pro Leu Lys Asn Gln Ala Lys Asn Ala Gly Thr Ser Met
    205                 210                 215

Asp Pro Ser Thr Thr Thr Phe Asp Asn Thr Tyr Tyr Arg Leu Ile Leu
220                 225                 230                 235

Gln Gln Lys Gly Leu Phe Ser Ser Asp Gln Val Leu Leu Asp Asn Pro
                240                 245                 250

Asp Thr Lys Asn Leu Val Thr Lys Phe Ala Thr Ser Lys Lys Ala Phe
            255                 260                 265

Tyr Glu Ala Phe Ala Lys Ser Met Ile Arg Met Ser Ser Tyr Asn Gly
        270                 275                 280

Gly Gln Glu Val Arg Arg Thr Ala Glu
    285                 290
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1167 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: 5'UTR
(B) LOCATION: 1..38

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 39..977

(ix) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 978..1167

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 39..101

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 102..977

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
GGCACGAGGC TAAAAATCAT CGAAGTACTC AAATTAGC ATG GCT GTC ATG GTT          53
                                          Met Ala Val Met Val
                                          -21 -20

GCA TTC TTG AAT TTG ATC ATC ATG TTT TCA GTA GTC TCT ACA AGC AAG        101
Ala Phe Leu Asn Leu Ile Ile Met Phe Ser Val Val Ser Thr Ser Lys
    -15                 -10                 -5

TCA CTG AGC TTA AAC TAC TAT TCA AAA ACA TGC CCT GAT GTG GAA TGC        149
Ser Leu Ser Leu Asn Tyr Tyr Ser Lys Thr Cys Pro Asp Val Glu Cys
  1               5                  10                  15

ATT GTT GCC AAG GCA GTG AAG GAT GCC ACT GCT AGG GAC AAA ACT GTT        197
Ile Val Ala Lys Ala Val Lys Asp Ala Thr Ala Arg Asp Lys Thr Val
            20                  25                  30

CCA GCT GCA CTT CTG CGA ATG CAC TTC CAT GAC TGT TTC GTT CGG GGG        245
Pro Ala Ala Leu Leu Arg Met His Phe His Asp Cys Phe Val Arg Gly
        35                  40                  45

TGT GGT GCC TCT GTG CTG CTA AAT TCA AAA GGA AGC AAC AAA GCA GAA        293
Cys Gly Ala Ser Val Leu Leu Asn Ser Lys Gly Ser Asn Lys Ala Glu
    50                  55                  60

AAA GAT GGG CCA CCA AAT GTT TCT TTG CAT GCA TTC TAT GTC ATT GAT        341
Lys Asp Gly Pro Pro Asn Val Ser Leu His Ala Phe Tyr Val Ile Asp
65                  70                  75                  80

GCA GCG AAG AAA GCA CTA GAA GCT TCA TGC CCA GGT GTG GTC TCT TGT        389
Ala Ala Lys Lys Ala Leu Glu Ala Ser Cys Pro Gly Val Val Ser Cys
                85                  90                  95

GCT GAC ATC CTT GCT CTA GCA GCA AGG GAT GCA GTT TTT CTG TCA GGA        437
Ala Asp Ile Leu Ala Leu Ala Ala Arg Asp Ala Val Phe Leu Ser Gly
            100                 105                 110

GGA CCT ACA TGG GAT GAA CCT AAA GGA AGA AAG GAT GGC AGA ACA TCT        485
Gly Pro Thr Trp Asp Glu Pro Lys Gly Arg Lys Asp Gly Arg Thr Ser
        115                 120                 125

AAA GCC AGC GAA ACC AGA CAA TTA CCA GCA CCA ACC TTC AAC TTA TCA        533
Lys Ala Ser Glu Thr Arg Gln Leu Pro Ala Pro Thr Phe Asn Leu Ser
    130                 135                 140

CAA CTG CGG CAA AGC TTT TCT CAA AGA GGA CTG TCA GGG GAA GAC CTG        581
Gln Leu Arg Gln Ser Phe Ser Gln Arg Gly Leu Ser Gly Glu Asp Leu
145                 150                 155                 160

GTA GCT CTG TCA GGG GGG CAC ACT TTG GGT TTC TCT CAC TGC TCA TCT        629
Val Ala Leu Ser Gly Gly His Thr Leu Gly Phe Ser His Cys Ser Ser
                165                 170                 175

TTC AAG AAC AGA ATC CAC AAC TTC AAT GCT ACA CAT GAT GAA GAC CCT        677
Phe Lys Asn Arg Ile His Asn Phe Asn Ala Thr His Asp Glu Asp Pro
```

```
            180                 185                 190

TCA TTA AAT CCA TCA TTT GCA ACA AAA CTG ATA TCA ATT TGT CCA CTA        725
Ser Leu Asn Pro Ser Phe Ala Thr Lys Leu Ile Ser Ile Cys Pro Leu
        195                 200                 205

AAA AAT CAG GCA AAA AAT GCA GGC ACC TCT ATG GAC CCT TCA ACA ACA        773
Lys Asn Gln Ala Lys Asn Ala Gly Thr Ser Met Asp Pro Ser Thr Thr
    210                 215                 220

ACT TTT GAT AAT ACA TAT TAC AGG TTG ATC CTC CAA CAG AAA GGC TTG        821
Thr Phe Asp Asn Thr Tyr Tyr Arg Leu Ile Leu Gln Gln Lys Gly Leu
225                 230                 235                 240

TTT TCT TCT GAT CAA GTT TTG CTT GAC AAC CCA GAC ACT AAA AAT CTG        869
Phe Ser Ser Asp Gln Val Leu Leu Asp Asn Pro Asp Thr Lys Asn Leu
                245                 250                 255

GTT GCG AAG TTT GCC ACC TCA AAA AAG GCT TTT TAT GAC GCT TTT GCA        917
Val Ala Lys Phe Ala Thr Ser Lys Lys Ala Phe Tyr Asp Ala Phe Ala
            260                 265                 270

AAG TCC ATG ATC AAA ATG AGT AGC ATC AAT GGT GGA CAG GAG GTT AGA        965
Lys Ser Met Ile Lys Met Ser Ser Ile Asn Gly Gly Gln Glu Val Arg
        275                 280                 285

AGG ACT GCA GAG TGATCAATTA AAAAGTCTTA AATTAATTCA AGTTAAATTG           1017
Arg Thr Ala Glu
    290

ATGTTTCAAA CAAGTTAGAA GTATGAACTT GTTGGATCAA ATTTCCTAGA TGGCAAGATA     1077

TTATGTCTTT TTCCTCTATT AAAGAAATAT GTATATTTAT CTGAAGTTAA TAAATATATC     1137

ATTTTGATAA AAAAAAAAAA AAAAAAAAAA                                      1167
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 313 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Met Ala Val Met Val Ala Phe Leu Asn Leu Ile Ile Met Phe Ser Val
-21 -20                 -15                 -10

Val Ser Thr Ser Lys Ser Leu Ser Leu Asn Tyr Tyr Ser Lys Thr Cys
 -5                  1                   5                  10

Pro Asp Val Glu Cys Ile Val Ala Lys Ala Val Lys Asp Ala Thr Ala
             15                  20                  25
```

```
Arg Asp Lys Thr Val Pro Ala Ala Leu Leu Arg Met His Phe His Asp
          30                  35                  40

Cys Phe Val Arg Gly Cys Gly Ala Ser Val Leu Leu Asn Ser Lys Gly
      45                  50                  55

Ser Asn Lys Ala Glu Lys Asp Gly Pro Pro Asn Val Ser Leu His Ala
 60                  65                  70                  75

Phe Tyr Val Ile Asp Ala Ala Lys Lys Ala Leu Glu Ala Ser Cys Pro
                 80                  85                  90

Gly Val Val Ser Cys Ala Asp Ile Leu Ala Leu Ala Ala Arg Asp Ala
             95                 100                 105

Val Phe Leu Ser Gly Gly Pro Thr Trp Asp Glu Pro Lys Gly Arg Lys
        110                 115                 120

Asp Gly Arg Thr Ser Lys Ala Ser Glu Thr Arg Gln Leu Pro Ala Pro
    125                 130                 135

Thr Phe Asn Leu Ser Gln Leu Arg Gln Ser Phe Ser Gln Arg Gly Leu
140                 145                 150                 155

Ser Gly Glu Asp Leu Val Ala Leu Ser Gly Gly His Thr Leu Gly Phe
                160                 165                 170

Ser His Cys Ser Ser Phe Lys Asn Arg Ile His Asn Phe Asn Ala Thr
            175                 180                 185

His Asp Glu Asp Pro Ser Leu Asn Pro Ser Phe Ala Thr Lys Leu Ile
        190                 195                 200

Ser Ile Cys Pro Leu Lys Asn Gln Ala Lys Asn Ala Gly Thr Ser Met
    205                 210                 215

Asp Pro Ser Thr Thr Thr Phe Asp Asn Thr Tyr Tyr Arg Leu Ile Leu
220                 225                 230                 235

Gln Gln Lys Gly Leu Phe Ser Ser Asp Gln Val Leu Leu Asp Asn Pro
                240                 245                 250

Asp Thr Lys Asn Leu Val Ala Lys Phe Ala Thr Ser Lys Lys Ala Phe
            255                 260                 265

Tyr Asp Ala Phe Ala Lys Ser Met Ile Lys Met Ser Ser Ile Asn Gly
        270                 275                 280

Gly Gln Glu Val Arg Arg Thr Ala Glu
    285                 290
```

Claims

WHAT IS CLAIMED IS:

1. An isolated DNA consisting essentially of cDNA coding for an *SEP*a1 polypeptide.

2. The isolated DNA of claim 1, wherein said *SEP*a1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO:11.

3. An isolated DNA consisting essentially of DNA having at least 15 nucleotides of the cDNA of claim 1.

4. An isolated DNA consisting essentially of cDNA coding for an *SEP*a2 polypeptide.

5. The isolated DNA of claim 4 wherein said *SEP*a2 polypeptide comprises the amino acid sequence set forth in SEQ ID NO:13.

6. An isolated DNA consisting essentially of DNA having at least 15 nucleotides of the cDNA of claim 4.

7. An isolated DNA consisting essentially of cDNA coding for an *SEP*b1 polypeptide.

8. The isolated DNA of claim 7 wherein said *SEP*b1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 15.

9. An isolated DNA consisting essentially of DNA having at least 15 nucleotides of the cDNA of claim 7.

10. An isolated DNA consisting essentially of cDNA coding for an *SEP*b2 polypeptide.

11. The isolated DNA of claim 10 wherein said *SEP*a1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 17.

12. An isolated DNA consisting essentially of DNA having at least 15 nucleotides of the cDNA of claim 10.

13. A pair of single-stranded DNA primers, wherein the sequence of said primers is derived from the sequence set forth in SEQ ID NO:10, wherein the use of

said primers in a polymerase chain reaction results in the synthesis of DNA having all or part of the sequence of the *SEP*a1 gene.

14. A pair of single-stranded DNA primers, wherein the sequence of said primers is derived from the sequence set forth in SEQ ID NO: 12, wherein the use of said primers in a polymerase chain reaction results in the synthesis of DNA having all or part of the sequence of the *SEP*a2 gene.

15. A pair of single-stranded DNA primers, wherein the sequence of said primers is derived from the sequence set forth in SEQ ID NO: 14, wherein the use of said primers in a polymerase chain reaction results in the synthesis of DNA having all or part of the sequence of the *SEP*b1 gene.

16. A pair of single-stranded DNA primers, wherein the sequence of said primers is derived from the sequence set forth in SEQ ID NO: 16, wherein the use of said primers in a polymerase chain reaction results in the synthesis of DNA having all or part of the sequence of the *SEP*b2 gene.

17. A nucleic acid probe complementary to *SEP*a1 gene sequences.

18. A nucleic acid probe complementary to *SEP*a2 gene sequences.

19. A nucleic acid probe complementary to *SEP*b1 gene sequences.

20. A nucleic acid probe complementary to *SEP*b2 gene sequences.

21. A replicative cloning vector which comprises the isolated DNA of any one of claims 1-3 and a replicon operative in a host cell.

22. A replicative cloning vector which comprises the isolated DNA of any one of claims 4-6 and a replicon operative in a host cell.

23. A replicative cloning vector which comprises the isolated DNA of any one of claims 7-9 and a replicon operative in a host cell.

24. A replicative cloning vector which comprises the isolated DNA of any one of claims 10-12 and a replicon operative in a host cell.

25. A replicative cloning vector which comprises the isolated DNA of any one of claims 13-20 and a replicon operative in a host cell.

26. An expression system which comprises the isolated DNA of any one of claims 1-3 operably linked to suitable control sequences.

27. An expression system which comprises the isolated DNA of any one of claims 4-6 operably linked to suitable control sequences.

28. An expression system which comprises the isolated DNA of any one of claims 7-9 operably linked to suitable control sequences.

29. An expression system which comprises the isolated DNA of any one of claims 10-12 operably linked to suitable control sequences.

30. An expression system which comprises the isolated DNA of any one of claims 13-20 operably linked to suitable control sequences.

31. Recombinant host cells transformed with the expression system of claim 26.

32. Recombinant host cells transformed with the expression system of claim 27.

33. Recombinant host cells transformed with the expression system of claim 28.

34. Recombinant host cells transformed with the expression system of claim 29.

35. Recombinant host cells transformed with the expression system of claim 30.

36. A method of producing recombinant *SEP*a1 polypeptide which comprises culturing the cells of claim 31 under conditions effective for the production of said *SEP*a1 polypeptide.

37. A method of producing recombinant *SEP*a2 polypeptide which comprises culturing the cells of claim 32 under conditions effective for the production of said *SEP*a2 polypeptide.

38. A method of producing recombinant *SEP*b1 polypeptide which comprises culturing the cells of claim 33 under conditions effective for the production of said *SEP*b1 polypeptide.

39. A method of producing recombinant *SEP*b2 polypeptide which comprises culturing the cells of claim 34 under conditions effective for the production of said *SEP*b2 polypeptide.

40. A preparation of soybean *SEP*a1 polypeptide substantially free of other soybean proteins, the amino acid sequence of said polypeptide corresponding to that shown in SEQ ID NO: 11.

41. A preparation of soybean *SEP*a2 polypeptide substantially free of other soybean proteins, the amino acid sequence of said polypeptide corresponding to that shown in SEQ ID NO: 13.

42. A preparation of soybean *SEP*b1 polypeptide substantially free of other soybean proteins, the amino acid sequence of said polypeptide corresponding to that shown in SEQ ID NO: 15.

43. A preparation of soybean *SEP*b2 polypeptide substantially free of other soybean proteins, the amino acid sequence of said polypeptide corresponding to that shown in SEQ ID NO: 17.

44. An antibody immunoreactive with a plant peroxidase polypeptide and not substantially immunoreactive with other plant polypeptides.

45. The antibody of claim 44, wherein said antibody does not interfere with the enzymatic active of said polypeptide when bound to said antibody.

46. The antibody of claim 44 which is a monoclonal antibody.

47. The antibody of claim 45 which is a monoclonal antibody.

48. A hybridoma which produces the monoclonal antibody of claim 46.

49. A hybridoma which produces the monoclonal antibody of claim 47.

50. A non-destructive assay for peroxidase activity in plant tissue which comprises a) extracting peroxidase from a small section of said plant tissue, b) contacting said extracted peroxidase with an antibody which is immunoreactive with said peroxidase and which does not interfere with the enzymatic activity of the peroxidase when bound to the antibody, and c) measuring the activity of the antibody bound peroxidase.

51. The assay of claim 50, wherein the plant tissue is seed coat.

52. The assay of claim 51 wherein the plant tissue is soybean, corn, sunflowers, wheat, sorghum, arabidopsis, peanuts, tomatoes, brassica, onion, potato, horseradish, radish and oats.

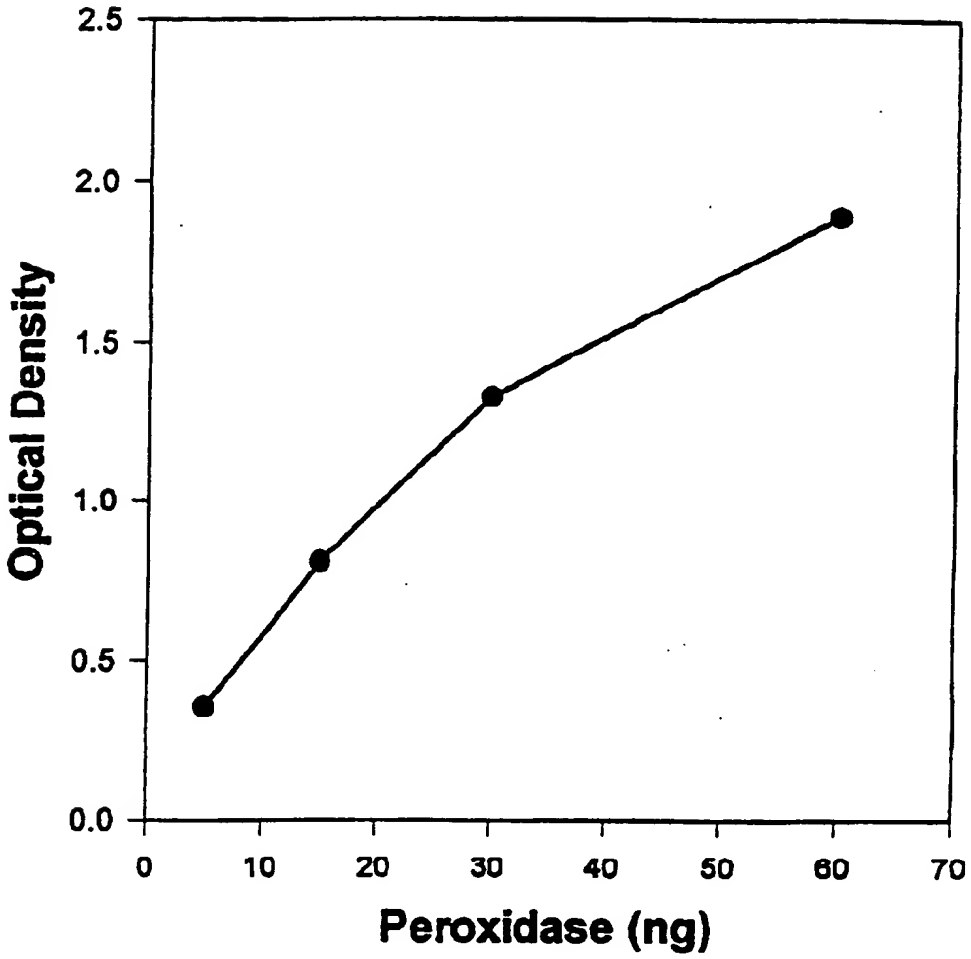

2.5
2.0
1.5
1.0
0.5
0.0
Optical Density
0
10
20
30
40
50
60
70
Peroxidase (ng)

Figure 1

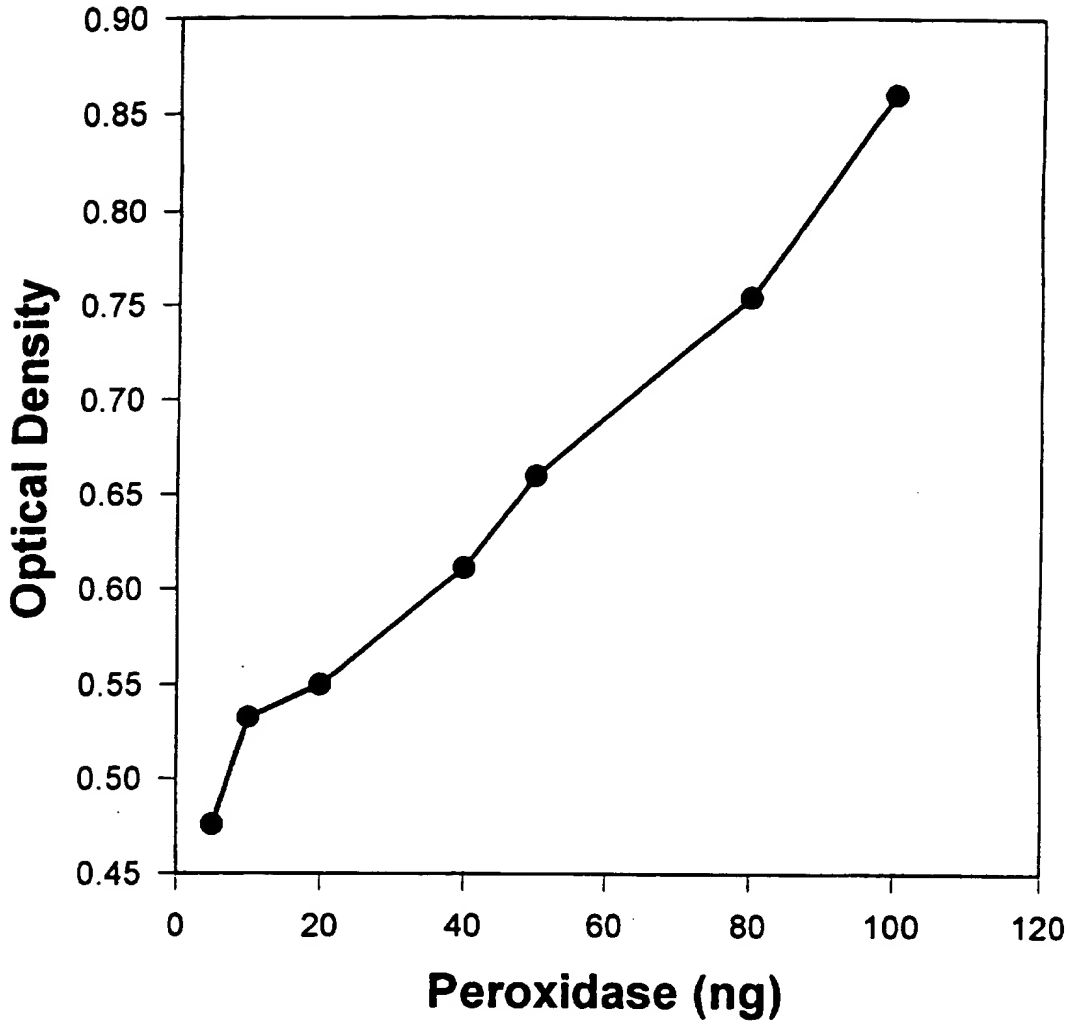

0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
Optical Density
0
20
40
60
80
100
120
Peroxidase (ng)

Figure 2

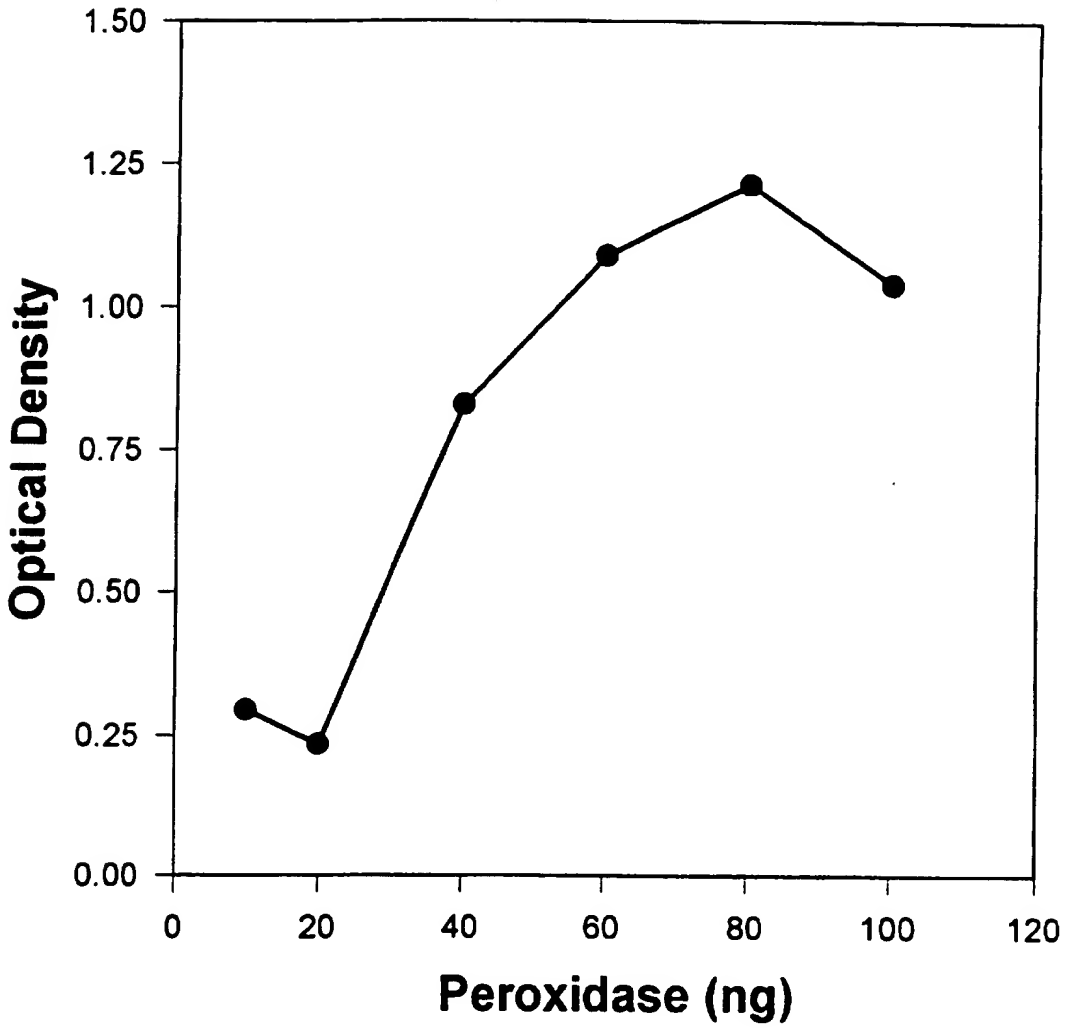

1.50
1.25
1.00
0.75
0.50
0.25
0.00
Optical Density
0
20
40
60
80
100
120
Peroxidase (ng)

Figure 3

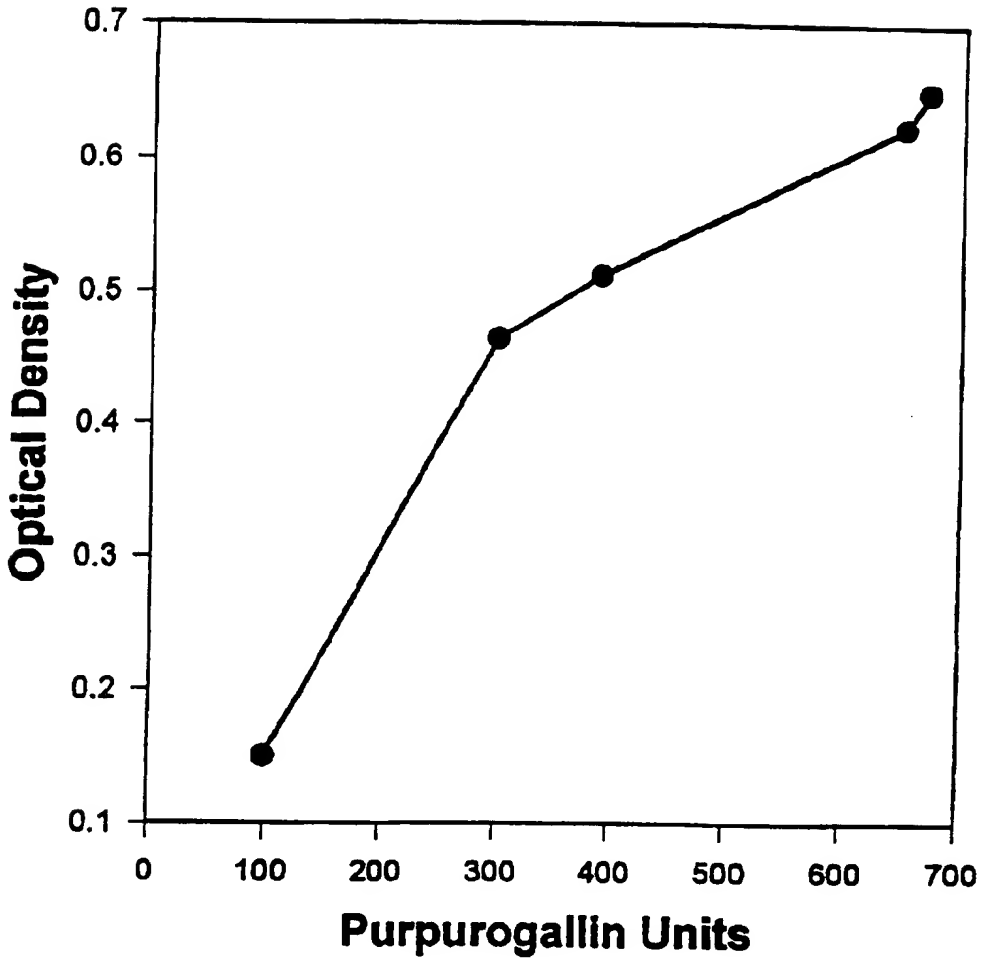

0.7
0.6
0.5
0.4
0.3
0.2
0.1
Optical Density
0
100
200
300
400
500
600
700
Purpurogallin Units

Figure 4

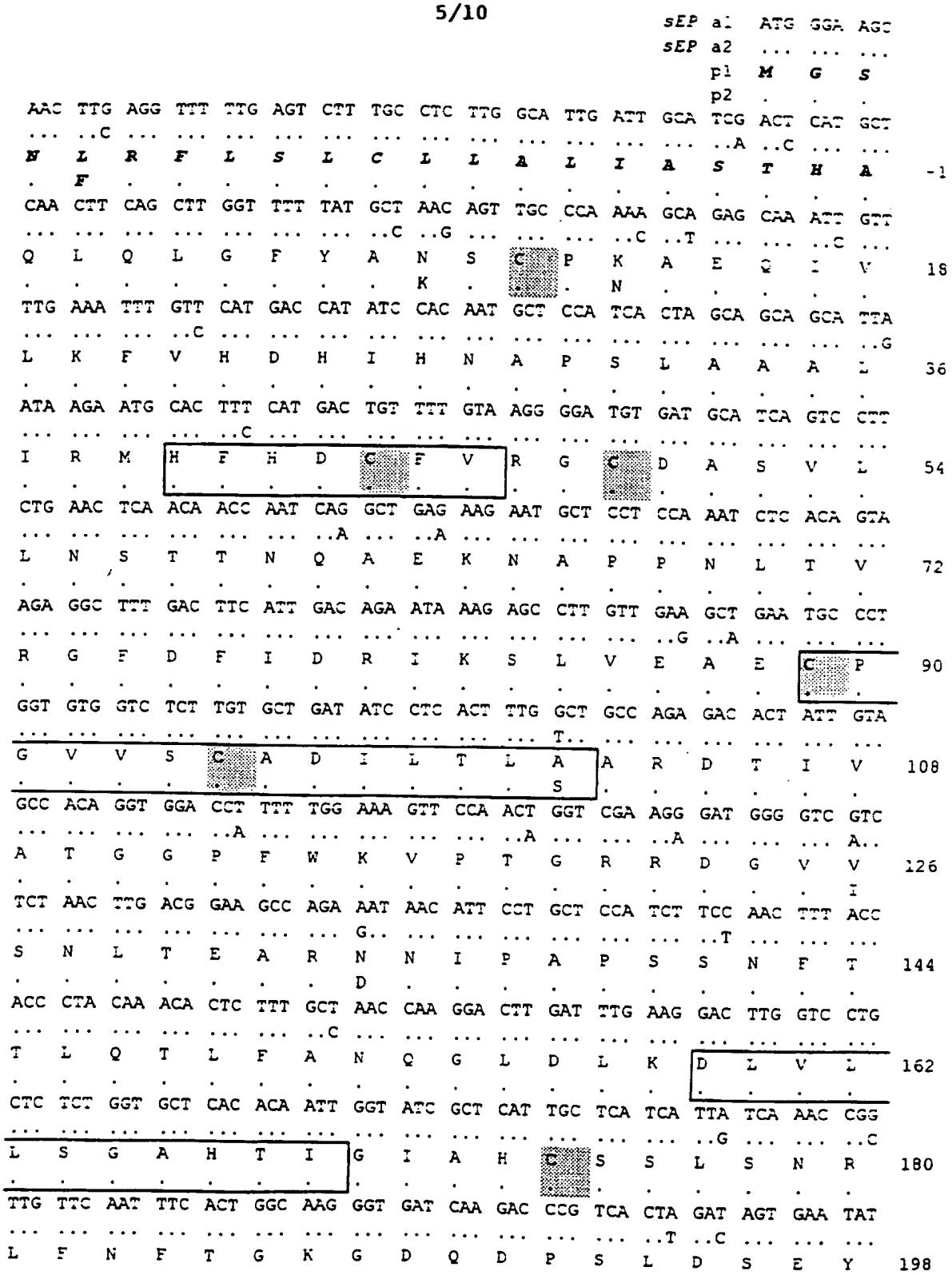
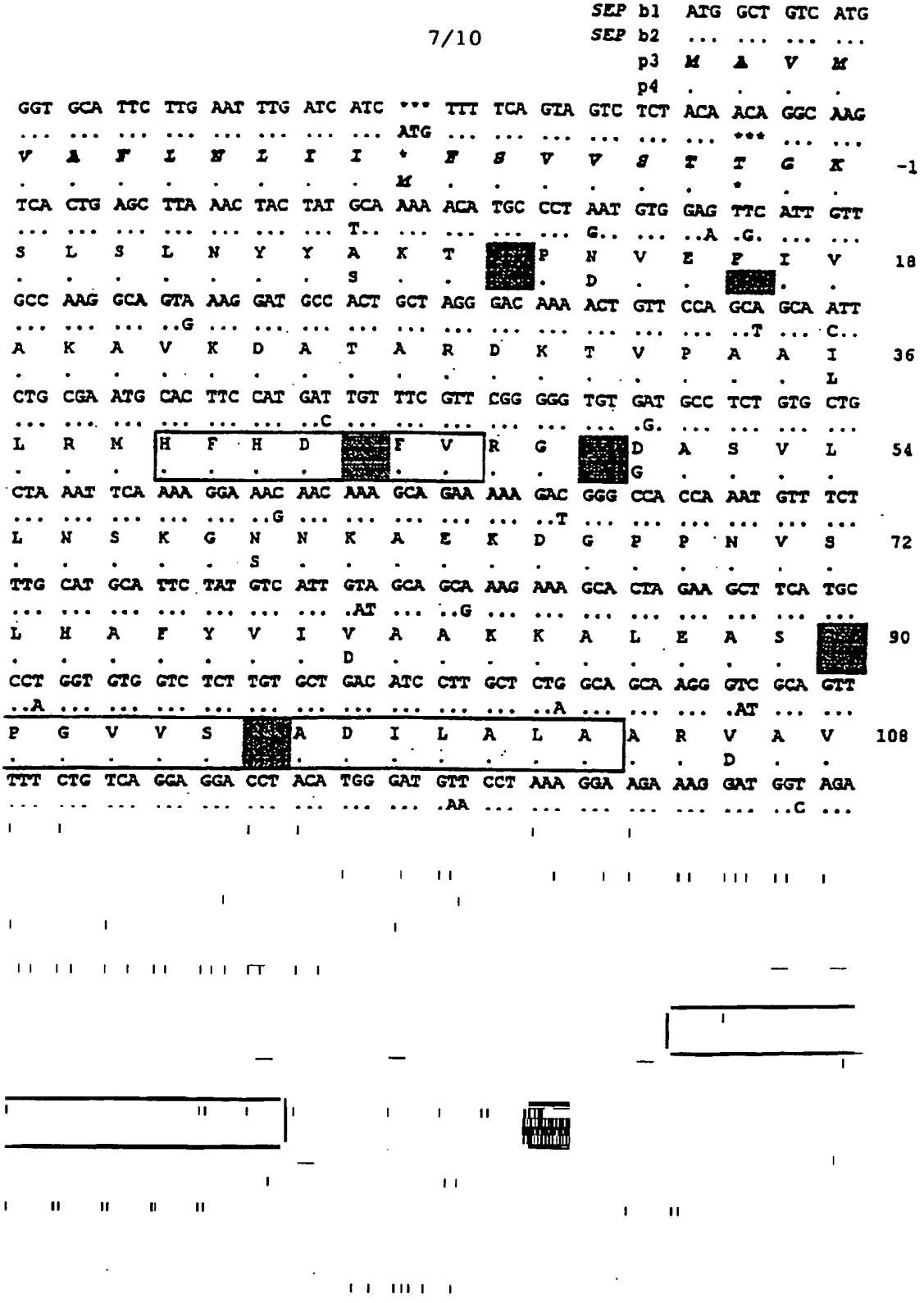

sEP a1 ATG GGA AGC
sEP a2 ... ...
p1 M G S
p2 . . .
AAC TTG AGG TTT TTG AGT CTT TGC CTC TTG GCA TTG ATT GCA TCG ACT CAT GCT
N L R F L S L C L L A L I A S T H A -1
CAA CTT CAG CTT GGT TTT TAT GCT AAC AGT TGC CCA AAA GCA GAG CAA ATT GTT
Q L Q L G F Y A N S C P K A E Q I V 18
TTG AAA TTT GTT CAT GAC CAT ATC CAC AAT GCT CCA TCA CTA GCA GCA GCA TTA
L K F V H D H I H N A P S L A A A L 36
ATA AGA ATG CAC TTT CAT GAC TGT TTT GTA AGG GGA TGT GAT GCA TCA GTC CTT
I R M H F H D C F V R G C D A S V L 54
CTG AAC TCA ACA ACC AAT CAG GCT GAG AAG AAT GCT CCT CCA AAT CTC ACA GTA
L N S T T N Q A E K N A P P N L T V 72
AGA GGC TTT GAC TTC ATT GAC AGA ATA AAG AGC CTT GTT GAA GCT GAA TGC CCT
R G F D F I D R I K S L V E A E C P 90
GGT GTG GTC TCT TGT GCT GAT ATC CTC ACT TTG GCT GCC AGA GAC ACT ATT GTA
G V V S C A D I L T L A A R D T I V 108
GCC ACA GGT GGA CCT TTT TGG AAA GTT CCA ACT GGT CGA AGG GAT GGG GTC GTC
A T G G P F W K V P T G R R D G V V 126
TCT AAC TTG ACG GAA GCC AGA AAT AAC ATT CCT GCT CCA TCT TCC AAC TTT ACC
S N L T E A R N N I P A P S S N F T 144
ACC CTA CAA ACA CTC TTT GCT AAC CAA GGA CTT GAT TTG AAG GAC TTG GTC CTG
T L Q T L F A N Q G L D L K D L V L 162
CTC TCT GGT GCT CAC ACA ATT GGT ATC GCT CAT TGC TCA TCA TTA TCA AAC CGG
L S G A H T I G I A H C S S L S N R 180
TTG TTC AAT TTC ACT GGC AAG GGT GAT CAA GAC CCG TCA CTA GAT AGT GAA TAT
L F N F T G K G D Q D P S L D S E Y 198

Figure 5A

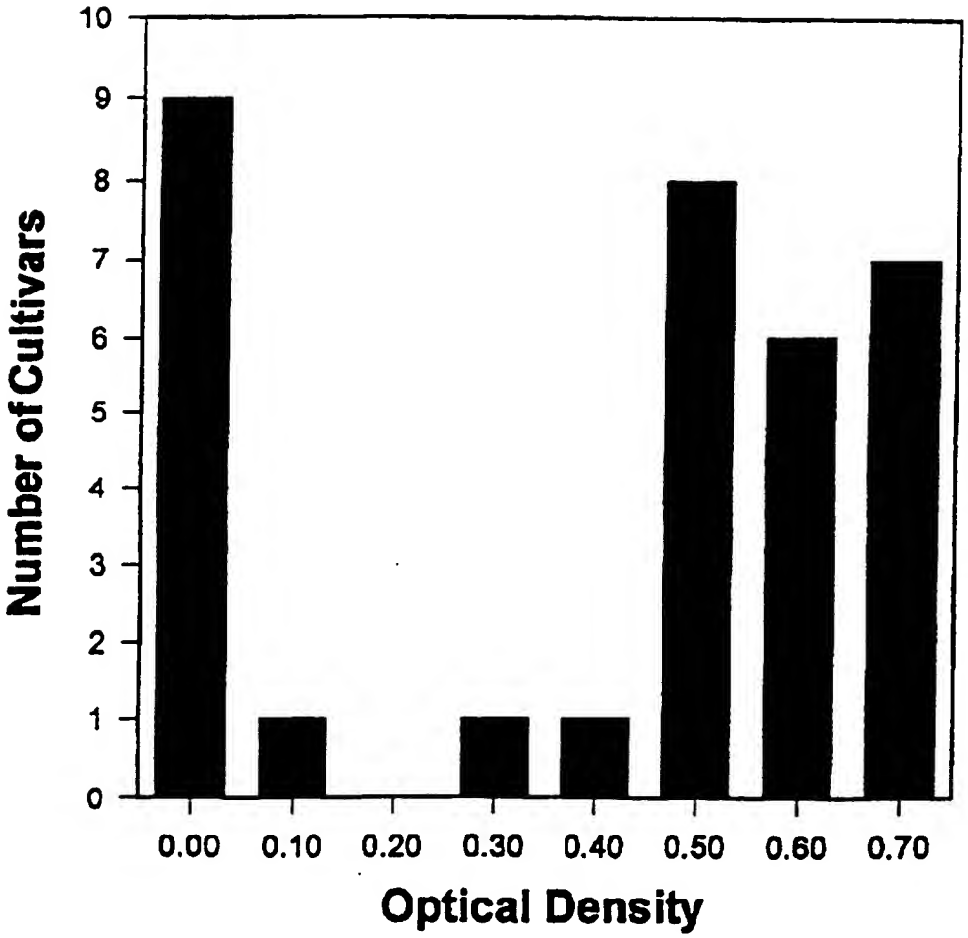
```
.   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
GCT GCA AAT TTG AAA GCA TTC AAG TGC ACA GAC CTC AAC AAG TTG AAC ACC ACA
... ... ... C.. ... ..C ... ... ... ..G ... ... ..T ... ... ... ... ...
A   A   N   L   K   A   F   K   C   T   D   L   N   K   L   N   T   T   216
.   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
AAA ATT GAG ATG GAC CCT GGA AGT CGC AAG ACA TTT GAT CTT AGC TAC TAT AGT
... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .   .
K   I   E   M   D   P   G   S   R   K   T   F   D   L   S   Y   Y   S   234
.   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
CAC GTT ATT AAG AGA AGG GGT CTA TTT GAG TCA GAT GCT GCA TTA TTG ACT AAC
... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ..A ...
H   V   I   K   R   R   G   L   F   E   S   D   A   A   L   L   T   N   252
.   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
TCA GTT ACA AAG GCA CAA ATC ATC CAA TTG CTT GAA GGG TCA GTT GAA AAT TTC
... ... ... ... .G. ... ... ..T ..G ... ... ... ... ... ... ... ... ...
S   V   T   K   A   Q   I   I   Q   L   L   E   G   S   V   E   N   F   270
.   .   .   .   .   .   .   .   E   .   .   .   .   .   .   .   .   .
TTT GCT GAG TTT GCA ACC TCC ATC GAG AAA ATG GGA AGA ATT AAT GTG AAG ACA
... ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ...
F   A   E   F   A   T   S   I   E   K   M   G   R   I   N   V   K   T   288
.   .   .   .   .   .   .   M   .   .   .   .   .   .   .   .   .   .
GGC ACA GAA GGA GAG ATC AGG AAG CAT TGT GCA TTT ATA AAT AGC TAA
..G ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ...
G   T   E   G   E   I   R   K   H   C   A   F   I   N   S   end 303
.   .   .   .   .   .   .   .   .   .   .   .   L   .   .   end
```

Figure 5B


SEP b1 ATG GCT GTC ATG
SEP b2
p3 M A V M
p4
GGT GCA TTC TTG AAT TTG ATC ATC *** TTT TCA GTA GTC TCT ACA ACA GGC AAG
V A F L N L I I * F S V V S T T G K -1
TCA CTG AGC TTA AAC TAC TAT GCA AAA ACA TGC CCT AAT GTG GAG TTC ATT GTT
S L S L N Y Y A K T P N V E F I V 18
GCC AAG GCA GTA AAG GAT GCC ACT GCT AGG GAC AAA ACT GTT CCA GCA GCA ATT
A K A V K D A T A R D K T V P A A I 36
CTG CGA ATG CAC TTC CAT GAT TGT TTC GTT CGG GGG TGT GAT GCC TCT GTG CTG
L R M H F H D F V R G D A S V L 54
CTA AAT TCA AAA GGA AAC AAC AAA GCA GAA AAA GAC GGG CCA CCA AAT GTT TCT
L N S K G N N K A E K D G P P N V S 72
TTG CAT GCA TTC TAT GTC ATT GTA GCA GCA AAG AAA GCA CTA GAA GCT TCA TGC
L H A F Y V I V A A K K A L E A S 90
CCT GGT GTG GTC TCT TGT GCT GAC ATC CTT GCT CTG GCA GCA AGG GTC GCA GTT
P G V V S A D I L A L A A R V A V 108
TTT CTG TCA GGA GGA CCT ACA TGG GAT GTT CCT AAA GGA AGA AAG GAT GGT AGA

```
GCA GCA AAA CTG ATC TCA ATT TGT CCA CTA AAA AAT CAG GCA AAA AAT GCA GGC
... A.. ... ... ..A ... .. ... ... ... ... ... ... ... ... ... ... ...
A   A   K   L   I   S   I   [redacted] P   L   K   N   Q   A   K   N   A   G   216
.   T   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
ACC TCT ATG GAC CCT TCA ACA ACA ACT TTT GAT AAT ACA TAT TAC AGG TTG ATC
... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
T   S   M   D   P   S   T   T   T   F   D   N   T   Y   Y   R   L   I   234
.   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
CTC CAA CAG AAA GGC TTG TTT TCT TCT GAT CAA GTT TTG CTT GAC AAC CCA GAC
... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
L   Q   Q   K   G   L   F   S   S   D   Q   V   L   L   D   N   P   D   252
.   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
ACT AAA AAT CTG GTT ACA AAG TTT GCC ACC TCA AAA AAG GCT TTT TAT GAG GCT
... ... ... ... ... G.G ... ... ... ... ... ... ... ... ... ..C ...
T   K   N   L   V   T   K   F   A   T   S   K   K   A   F   Y   E   A   270
.   .   .   .   .   A   .   .   .   .   .   .   .   .   .   D   .
TTT GCG AAG TCC ATG ATC AGA ATG AGT AGC TAC AAT GGT GGA CAG GAG GTT AGA
... ... ... ... ... ... .A. ... ... ... AT. ... ... ... ... ... ... ...
F   A   K   S   M   I   R   M   S   S   Y   N   G   G   Q   E   V   R   288
.   .   .   .   .   .   K   .   .   .   I   .   .   .   .   .   .   .   .
AGG ACT GCA GAA TGA 292
... ... ... ..G ...
R   T   A   E   end
.   .   .   .   end
```

Figure 6b


Number of Cultivars
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9
8
7
6
5
4
3
2
1
0
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
Optical Density

Figure 7

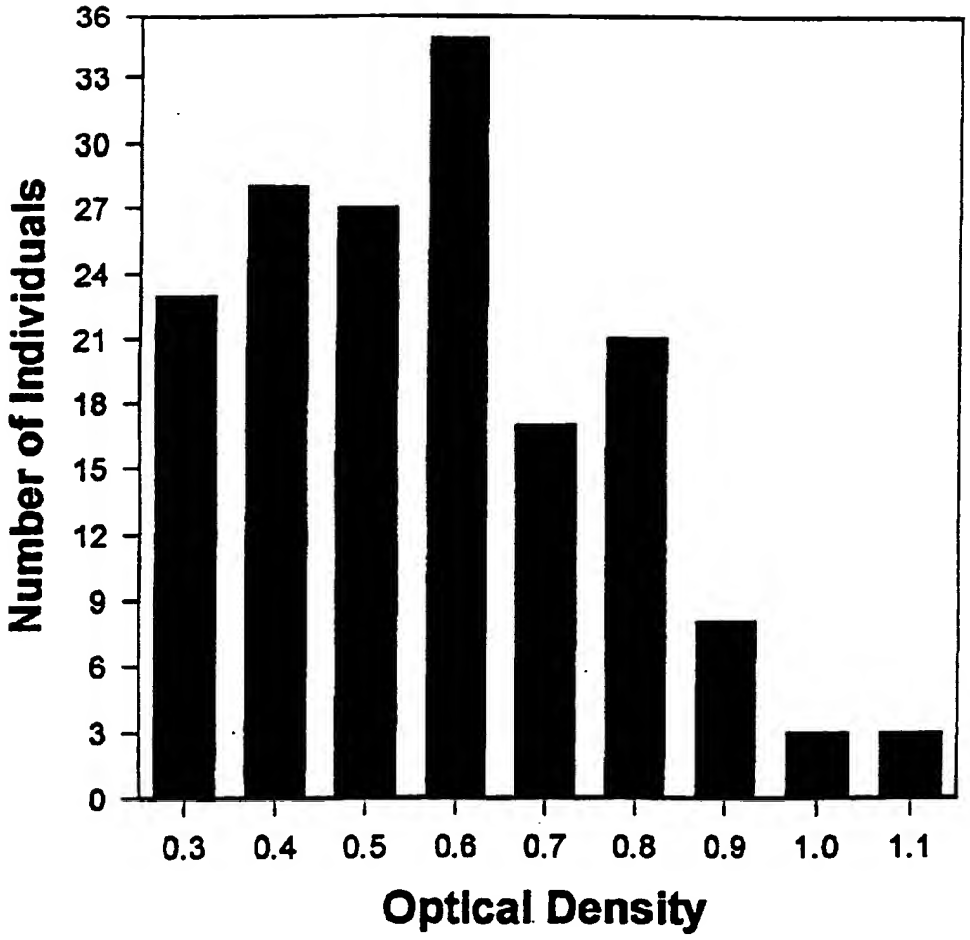
Number of Individuals
36
33
30
27
24
21
18
15
12
9
6
3
0
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
Optical Density

Figure 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16354

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 5/12, 9/00, 15/09, 15/29, 15/52, 15/63

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 69.1, 70.1, 172.3, 240.1, 240.27, 320.1; 530/378, 387.9, 388.26; 536/23.6, 24.3, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DALTON et al. Isolation and characterization of the gene for soybean cytosolic ascorbate peroxidase. Plant Physiology. 1994, Vol. 105, 1 Supplement, page 152, see entire document.	1-39
Y	ECKES et al. Overproduction of alfalfa glutamine synthetase in transgenic tobacco plants. Mol. Gen. Genet. 1989, Vol. 217, pages 263-268, especially pages 263-268, see entire document.	21-39
Y	US 5,112,752 A (JOHNSON et al) 12 May 1992, columns 11-14.	40-52
Y	SCHARFF et al. Hybridomas as a source of antibodies. Hospital Practice. January 1981, pages 61-66, see entire document.	44-49

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 JANUARY 1997	Date of mailing of the international search report 0 3 FEB 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ELIZABETH F. MCELWAIN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16354

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/7.1, 69.1, 70.1, 172.3, 240.1, 240.27, 320.1; 530/378, 387.9, 388.26; 536/23.6, 24.3, 24.33